Investigation of the Linkage between Gaucher Disease and B-Cell Disorders including Multiple Myeloma

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Thesis submitted for examination of Doctor of Philosophy (PhD)

January 2011

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I Robert Michael Ayto confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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ABSTRACT

Aim. To investigate the association between Gaucher disease (GD) and gammopathy. **Introduction**. Gaucher disease is a disorder characterised by deficiency in lysosomal glucocerebrosidase, foamy macrophages, cytopenias, bony lesions, organomegaly and a high incidence of monoclonal/polyclonal gammopathy. Abnormalities in the bone marrow microenvironment, including pseudo-Gaucher cells, have been reported in non-GD patients with myeloma. **Methods.** Gaucher gene analysis, co-culture, drug assays, western blotting, enzymatic assays, flow cytometry, immunofluorescence and cytotoxic assays. **Results.** GD patients had a high incidence of gammopathy (polyclonal/monoclonal). Serum biomarkers of macrophage burden were predictive of gammopathy and the Zimran severity score was higher in those with monoclonal than polyclonal gammopathy. Enzyme replacement therapy ameliorated polyclonal gammopathy and stabilised paraprotein levels. Gaucher mutations were not prevalent in Jewish patients with paraproteinaemia (8/77 patients). Non-GD patients with paraproteinaemia had normal serum chitotriosidase and monocyte glucocerebrosidase activity. GD monolayers (osteoclasts/macrophages) did not confer a proliferative or survival advantage on co-cultured myeloma cell lines. GD monolayers, compared to control cultures, reduced sensitivity to melphalan and this was contact dependent. Western blotting identified differences in the levels of Bim and Bcl-xL between myeloma cells harvested from control and GD monolayers. When cultured alone, GD monolayers generated more osteoclasts and this was enhanced by plasma cell co-culture (contact independent). GD monolayers did not preferentially rescue myeloma cells pre-treated with doxorubicin from cell death. Patients with GD have decreased lymphocyte glucocerebrosidase activity, abnormal lipid trafficking and low peripheral NK (natural-killer), invariant NK and CD4+ve T-cell numbers. Invariant NK cells, GD derived, displayed impaired proliferation to α-galactosylceramide. Cytotoxicity assays, derived from the GD peripheral blood mononuclear cells, displayed inferior killing compared to control assays. NK killing assays were equivalent between controls and GD patients. **Conclusion.** This thesis presents novel data, which may confer an increased risk of gammopathy in GD.

ACKNOWLEDGEMENTS

First and foremost I would like to thank my supervisors Dr Derralynn Hughes and Professor Atul Mehta for their guidance, financial support and supervision during my PhD project. I am grateful to Myeloma UK for co-funding this project. Without the support of participating patients, the Gaucher association and the nurses in the Lysosomal Storage Disorders Unit, this project would not have been possible.

There are many people who I would like to thank and certainly more than I can mention. These include Matthew Reed, Robert Baker and Adele Holmes for providing me with an introduction to laboratory techniques. In particular, Matthew and Robert were immensely helpful in establishing and troubleshooting investigative models. Furthermore I would like to thank Janet North and Dr Mark Lowdell for their expertise and providing access to equipment. In addition, I would like to extend gratitude to Dr Andy Steele and Dr Stan Wickremasinghe, for their support, company and friendship.

My parents, Pat and Brian, have supported me at every step of my career, with encouragement and generosity. I would like to dedicate this thesis to my family and my wife, Shireen, without whose support and understanding, writing of this thesis would have been intolerable.

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ABBREVIATIONS

αGalCer	Alpha-galactosylceramide
αMEM	Minimum essential medium eagle alpha modification
ACP	Acid phosphatase
ADP	Adenosine diphosphate
AML	Acute myeloid leukaemia
ASM	Asymptomatic myeloma
ASP	Aspirate
ATP	Adenosine triphosphate
ALC	Absolute lymphocyte count
ALL	Acute lymphoblastic leukaemia
APCs	Antigen presenting cells
APRIL	A proliferation-inducing ligand
ASH	American Society of Haematology
BAFF	B-cell activating factor
BCF	Barnet and Chase Farm
Bcl-2	B-cell lymphoma-2
BCR-ABL	Breakpoint cluster region-Abelson
BHR	Barking, Havering and Redbridge
BMB	Bone marrow burden
BODIPY	Boron-dipyrromethene
Bp	Base pairs
BMT	Bone marrow transplantation
BRCA1	Breast cancer type 1 susceptibility protein
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
CCL-18	Chemokine (C-C motif) ligand 18
CD	Cluster of differentiation
CDT	Cyclophosphamide, dexamethasone and thalidomide
CFSE	Carboxyfluorescein succinimidyl ester
CFU-GM	Granulocyte macrophage colony-stimulating factor
CM	Conditioned medium

CML	Chronic myeloid leukaemia
COX	Cyclooxygenase
CR	Complement Receptor
CSF-1	Colony stimulating factor-1
CT	Computer tomography
CTL	Cytotoxic T-lymphocytes
CVAD	Cyclophosphamide, vincristine, adriamycin and dexamethasone
DC	Dendritic cell
DKK-1	Dickkopf-related protein 1
DLBCL	Diffuse large B-cell lymphoma
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphates
DPX	Di-N-Butyl Phthalate in Xylene
EBV	Epstein-Barr virus
ECL	Enzymatic Chemiluminescence
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
ESR	Erythrocyte sedimentation rate
ERT	Enzyme replacement therapy
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FDGLU	Fluorescein di-β-D-glucopyranoside
FITC	Fluorescein isothiocyanate
FISH	Fluorescent in situ hybridization
FSC	Forward side scatter characteristics
GBA1	Glucocerebrosidase gene
GBAP	Glucocerebrosidase pseudogene
GD	Gaucher disease

GI	Gastro-intestinal
GM-CSF	Granulocyte macrophage colony-stimulating factor
HBSS	Hank's buffered salt solution
HCC	Hepatocellular carcinoma
HCL	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGF	Hepatocyte growth factor
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HMW	High molecular weight
HRP	Horseradish peroxidase
IC_{50}	The half maximal inhibitory concentration (IC50)
ICAM	Inter-cellular adhesion molecule
ICD	International classification of diseases
ICGG	International Collaborative Gaucher Group (ICGG)
IFN-γ	Interferon gamma
iGB3	Isoglobotrihexosylceramide
IL	Interleukin
IMWG	International Myeloma Working Group
iNKT cells	Invariant natural killer T cells
ISS	International staging system
LAMP	Lysosomal associated membrane protein
LDH	Lactate dehydrogenase
LPL	Lympho-plasmacytic lymphoma
LPS	Lipopolysaccharide
LSD	Lysosomal storage disorder
M6P	Mannose-6-phosphate
M1	Classically activated macrophages
M2	Alternatively activated macrophages
MACs	Magnetic-activated cell sorting
MAPK	Mitogen-activated protein (MAP) kinases
Mcl-1	Myeloid cell leukaemia-1
M-CSF	Macrophage colony-stimulating factor
MDR	Multi-drug resistance

MDS	Myelodysplastic syndrome
MFI	Median fluorescent intensity
MGUS	Monoclonal gammopathy of undetermined significance
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
ML	Mucolipidosis
MM	Multiple Myeloma
MPS	Mucopolysaccharidosis
MPT	Melphalan, prednisolone and thalidomide
MREC	Multi Centre Research Ethics Committee
MRI	Magnetic resonance imaging
MSCs	Mesenchymal stromal cells
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	Dimethyl thiazolyl diphenyl tetrazolium salt
MTXP	Metaxin 1 pseudogene
MU	Methylumbelliferone
MW	Mann-Whitney
NCBI	National Centre for Biotechnology Information
NCL	Neuronal Ceroid Lipofuscinoses
NICE	National Institute of Clinical Excellence
NF-κB	Nuclear factor κ B
NHL	Non-Hodgkin's lymphoma
NHS	National Health Service
NK	Natural killer
NR	Not reported
NSAIDs	Non-steroidal anti-inflammatory drugs
NWLH	North West London Hospitals
OC	Osteoclast
OPG	Osteoprotegerin
PARC	Pulmonary and activation-regulated chemokine
PARP	Poly (ADP-ribose) polymerase
PBMC/PBMCs	Peripheral blood mononuclear cell/cells
PBS	Phosphate buffered saline

PBSA	Phosphate buffered saline plus bovine serum albumin
PCR	Polymerase chain reaction
PD	Parkinson's disease
PE	Phycoerythrin
PerCP	Peridinin chlorphyll protein
PET	Positron emission tomography
PI	Propidium iodide
PKC	Protein kinase C
PopD	Population doubling
PR	Partial remission
PS	Phosphatidylserine
PSAP	Prosaposin
PTH	Parathyroid hormone
PUMA	*p53* upregulated modulator of apoptosis
RA	Rheumatoid arthritis
RANK	Receptor activator of nuclear factor κ B
RANK-L	Receptor activator of nuclear factor κ B ligand
RBC	Red blood cell
RFH	Royal Free Hospital
RNA	Ribonucleic acid
ROTI	Related organ or tissue impairment
RPMI	Roswell Park Memorial Institute medium
RR	Relative risk
RT-PCR	Reverse transcription polymerase chain reaction
S-ACE	Serum-angiotensin converting enzyme
SCID	Severe combined immunodeficiency
SD	Standard deviation
SAP	Serum amyloid P component
SRT	Substrate reduction therapy
SSC	Side scatter characteristics
STAT	Signal Transducers and Activators of Transcription protein
TAM	Tumour-associated macrophage
TCR	T-cell receptor
TEM	Transmission electron microscopy

THBS3	Thrombospondin-3
TLR	Toll-like receptor
TNFα	Tumour Necrosis Factor alpha
TRAIL	TNF-related apoptosis-inducing ligand
TRAP	Tartrate Resistant Acid Phosphatase
Tris	tris(hydroxymethyl)aminomethane
TW	Transwell
UK	United Kingdom
US	United States
USA	United States of America
VCAM	Vascular cell adhesion molecule
VLA	Very Late Antigen
ZSS	Zimran Severity Score

1 CHAPTER 1 – INTRODUCTION

1.1 LYSOSOMES – AN INTRODUCTION

Christian de Duve, discovered lysosomes in 1955, using cell fractionation, work for which he subsequently was awarded the Nobel Prize[1]. These organelles, only constitute about 0.5% of the cell volume of fibroblasts, but are much more abundant in macrophages[2]. Lysosomes, membraned organelles, are found in all cells accept for red blood cells[2]. They have important regulatory roles in the digestion of cellular proteins, catabolism of abnormal proteins, digestion of pathogenic micro-organisms, apoptosis, antigen presentation, degradation of intra-cellular organelles and the re-cycling of cell membrane constituents including receptors, sphingolipids and glycosaminoglycans (see Figure 1-1)[1-5]. In addition, packaged enzyme from the lysosome, can be transported by budded vesicles to the cell surface and undergo exocytosis, influencing a variety of disease processes, including the invasive properties of cancer cells[6;7]. Material is delivered to lysosomes for degradation via phagocytosis (phagosomes or autophagosomes), by clathrin-coated pits derived from the cell membrane (receptor mediated endocytosis) or ingested from the local microenvironment[2;8]. Intact lysosomal function is important for the degradation of bone and innate immunity in osteoclasts and neutrophils respectively[2]. Lysosomal related organelles include melanosomes and lytic granules in cytotoxic T-cells[2;8].

Lysosomes contain a cocktail of catabolic enzymes including lipases, proteases, glycosidases, phosphatases, sulfatases, and nucleases. Metabolised macromolecules, including amino acids, nucleotides, oligosaccharides and monosaccharides, are re-cycled back to the cytosol[2]. Due to the high metabolic activity of these organelles, lysosomal hydrolases are constantly replenished by newly synthesised enzyme and packaged for delivery from the Golgi apparatus following post-translational modification[9]. Lysosomal delivery of vesicle-packaged enzyme from the Golgi apparatus to the lysosomal compartment is facilitated by the addition of Mannose-6-phosphate (M6P) residues[9]. The lysosomal microenvironment is acidic, aiding dissociation of M6P residues from delivered hydrolases, provides an optimal pH for catalytic activity and denatures protein undergoing degradation[2]. Lysosomal

membranes consist of a heavily glycosylated glycolcalyx, lysosomal associated membrane proteins (LAMPs) and transmembrane transporters[2].

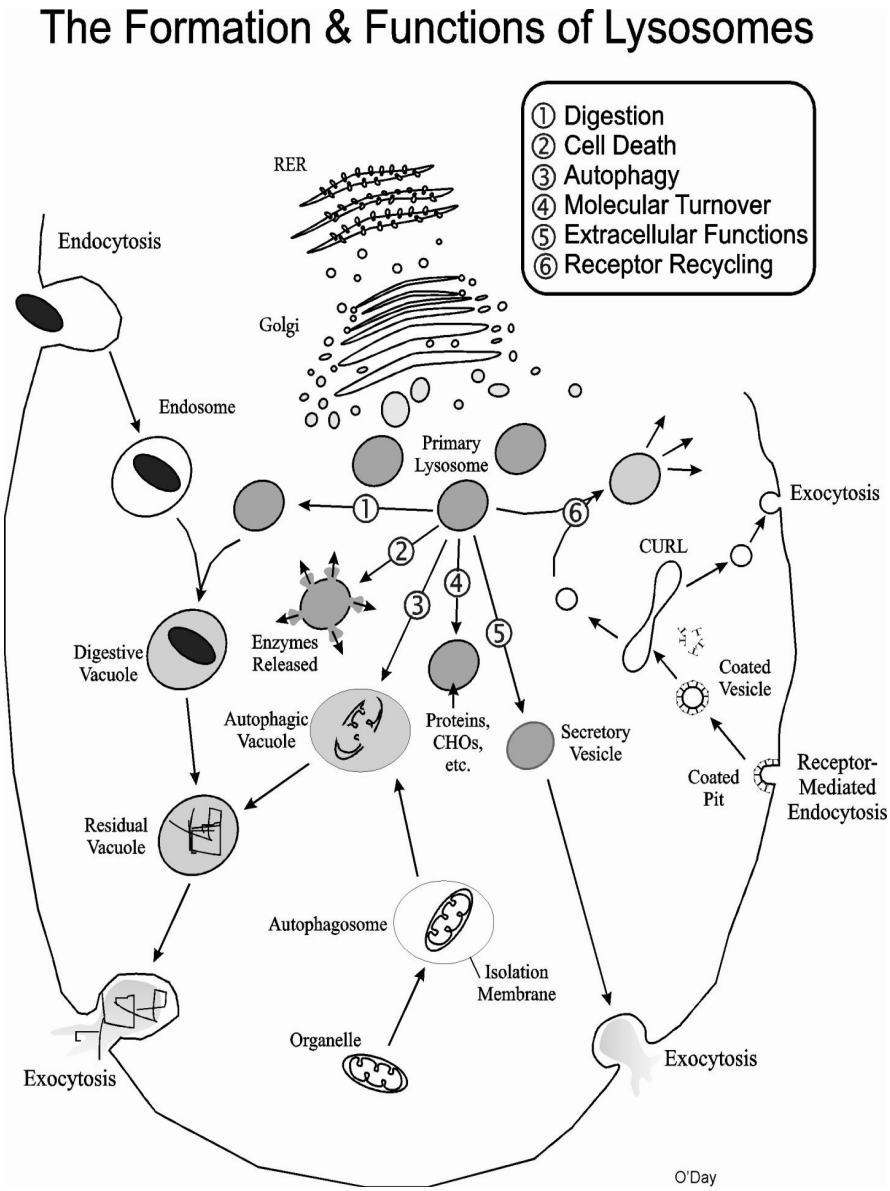


Figure 1-1. Schematic diagram of lysosomal function in mammalian cells.
Source:- www.utm.utoronto.ca/~w3bio315/lecture15.htm

1.1.1 LYSOSOMAL STORAGE DISORDERS

Currently, there have been described approximately 50 inherited deficiencies of metabolic enzymes or lysosomal-associated proteins that lead to clinical disease[5;10]. The lysosomal storage disorders (LSDs) are a heterogeneous group of diseases that most commonly occur from deficiency in a catabolic lysosomal enzyme. Hers and colleagues in the 1960s were the first to link deficiency in a lysosomal enzyme to the accumulation of substrate and lysosomal pathology (Pompe disease)[11]. Based on the

biochemical classification of the accumulated substrate the disorders could be subdivided into the sphingolipidoses (e.g. Gaucher disease and Fabry disease), mucopolysaccharidoses (e.g. Hurler's or Hunter's disease), glycoproteinoses (e.g. β-mannosidosis disease) and glycogenosis (e.g. Pompe disease) [5]. This classification is limited by the fact that many lysosomal enzymes are not specific for one substrate, such as in GM1 gangliosidosis, where accumulation of sphingolipids, keratin sulphate and oligosaccharides occurs due to low β-galactosidase activity[5]. In addition, due to the saturation of downstream enzymes, secondary substrates accumulate[3]. Enzyme deficiency compromises cells and organs where turnover of that specific substrate is high[10;12]. For example glycogen storage in the muscle of individuals with Pompe disease (α-maltase deficiency) or sphingolipid accumulation in macrophages involved in the digestion of apoptotic haematological cells in patients with Gaucher disease (glucocerebrosidase deficiency).

Many degradative enzymes, especially those involved in sphingolipid metabolism, require the presence of activator proteins, such as the saposins, that act as detergents, for physiological activity[5]. Mutations in saposin C (prosaposin gene, *PSAP*; codes for saposin A, B, C and D) have been reported to lead to a phenotype of Gaucher disease (GD) with normal β-glucocerebrosidase activity[13]. Physiological glucosylceramide metabolism requires the interaction of saposin C with glucocerebrosidase. Furthermore, deficiency in prosaposin and saposin B, present with clinical features consistent with neurovisceral dystrophy or metachromatic leukodystrophy respectively, due to non-physiological sphingolipid accumulation[14]. In addition, genetic disorders leading to abnormalities in transmembrane transport within the lysosomal membrane, including the cystine and sialic acid transporter, result in human disease[15]. An example of a deficiency of LAMP expression causing human pathology is Danon disease, a glycogen storage disorder characterised by the triad of heart disease, skeletal myopathy and mental retardation[2].

Cellular pathology secondary to deficiency in an individual lysosomal enzyme has been postulated to occur via several different mechanisms. These include the deposition of accumulated substrate (lysosomal and non-lysosomal), generalised lysosomal dysfunction, increased cellular concentrations of downstream substrates, decreased cellular concentrations of upstream metabolites, impaired calcium

homeostasis, endoplasmic reticulum (ER) stress, oxidative stress, free radical production, an up-regulation of pro-inflammatory cytokines, abnormal lipid trafficking, non-physiological signal conduction, altered auto-immunity, abnormal sphingolipid signalling and disturbed autophagy[3]. Mutant alleles in Gaucher disease, due to abnormal protein folding, are retained in the endoplasmic reticulum, induce ER stress and disturb normal cell physiology (see section 1.2.6)[16]. Abnormal calcium haemostasis has been shown in mouse models of GD in response to glucosylceramide and in human tissue from those with neuronopathic GD[17]. Ong *et al*. (2010) demonstrated that a reduction in ER calcium was detrimental to residual glucocerebrosidase activity due to a microenvironment skewed to the abnormal folding and retention of mutant alleles in the ER. GD fibroblasts have been shown to be more susceptible to oxidative stress[17].

The majority of LSDs are autosomal recessive in inheritance although several disorders, including Fabry disease (α-galactosidase deficiency), are X-linked[15]. In a retrospective Australian study carried out over a period of 16 years, Gaucher disease (GD) was reported in approximately 1:60,000 individuals and is the commonest LSD[18]. GD is characterised by disordered sphingolipid metabolism and like several other LSDs may present with neuronopathic disease. Storage disorders associated with central nervous system manifestations, including metachromatic leukodystrophy, neuronopathic GD and neuronal ceroid lipofuscinosis, confer a poor prognosis typified by early-onset death.

1.1.2 SPHINGOLIPIDOSIS

These comprise a group of disorders that are involved in the catabolism of sphingomyelins and glycosphingolipids (the cerebrosides, sulfatides, globosides and gangliosides). GD, Niemann Pick, Fabry disease and Tay-Sachs disease (see Figure 1-2) are examples of disorders secondary to sphingolipid accumulation[5]. Sphingolipids are composed of long-chain bases, fatty acids and a serine backbone[19]. They are an integral component of all cell membranes, regulate the activity of transmembrane receptors, manipulate immune response, including invariant natural killer T cell (NK-T) expansion, modulate host-pathogen interactions and are important in cell signalling[19-21].

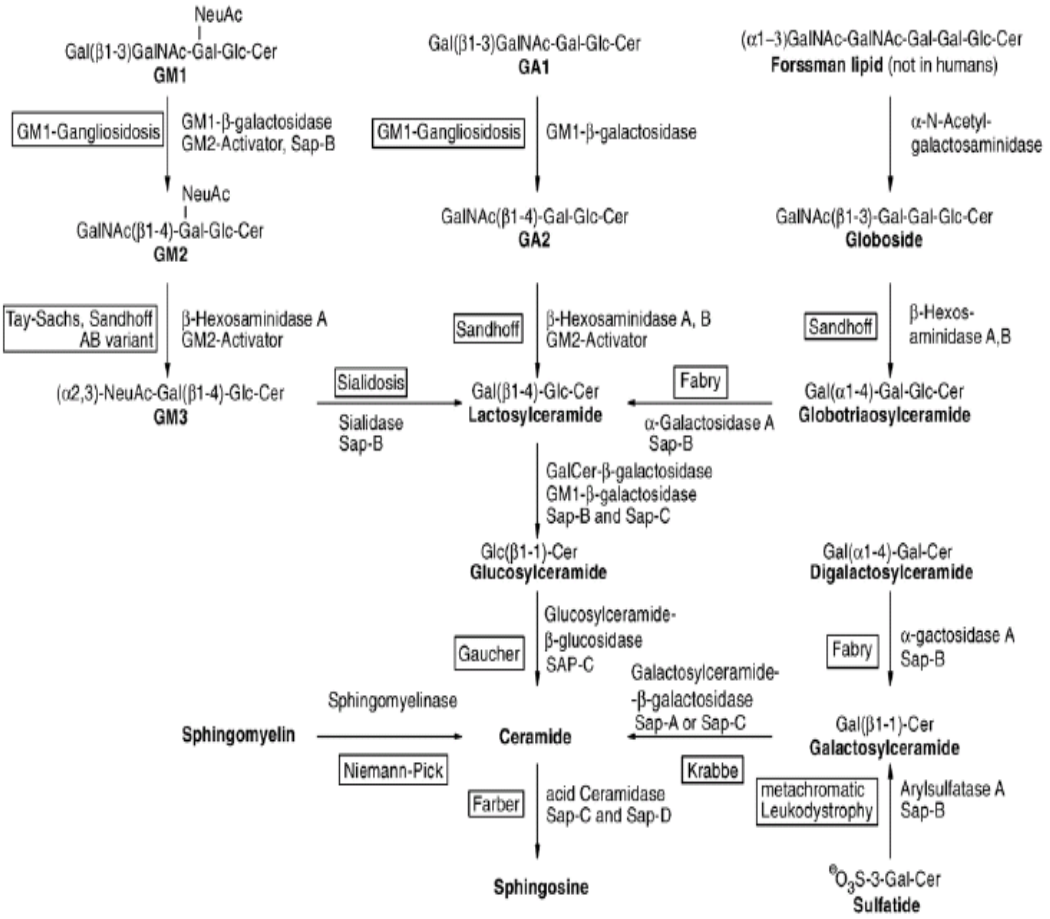


Figure 1-2. Sphingolipid metabolic pathway and inherited deficiencies leading to lysosomal dysfunction.
Source:- Sphingolipid metabolism disorders. Kolter and Sandhoff (2006)[22].

1.2 GAUCHER DISEASE – INTRODUCTION

Philippe Gaucher in 1882, whilst still a medical student, provided the first descriptions of this disorder in a young female with splenomegaly. Brady *et al.* (1965) described lysosomal β-glucocerebrosidase to be the deficient enzyme in GD[23]. Deficiency in this enzyme, an intermediate enzyme in sphingolipid metabolism, leads to accumulation of its substrate, glucosylceramide, within macrophages of the reticulo-endothelial system[24]. GD is an autosomal recessive disorder and is the commonest encountered LSD[18]. The gene coding for β-glucocerebrosidase (*GBA1*) was located to chromosome 1q21[25] in the late 1980s and was subsequently sequenced by Horowitz *et al.* (1989)[26]. To date, approximately 300 mutations of the

GBA1 allele have been reported[27]. Meikle *et al.* (1999) reported the incidence of GD, based on screening data from an Australian population, to be 1:57,000[18]. However, the true incidence of GD is not known, with estimates of between 1:40,000 to 1:100,000 being cited. Ethnicity data from an international Gaucher register in 1998 (recorded for 514 patients of the available 1,698 patients) identified 68% as being of Jewish descent (Ashkenazi and Sephardic combined)[28]. GD is more common in Ashkenazi Jews where incidence has been cited as ~1:900-1:1225, with a carrier frequency of between ~1:10-1:17.5[29;30].

1.2.1 *GBA1* GENE AND PSEUDOGENE

The *GBA1* gene is located on chromosome 1q21, consisting of 11 exons and 10 introns[27]. In 1989, Horowitz *et al.* sequenced the *GBA1* locus (7.6 kilobases pairs, kb) together with the 5.7kb pseudogene. Glucocerebrosidase cDNA is about 2kb in length and is translated into a 497-residue protein[27]. The pseudogene (*GBAP,* glucocerebrosidase A pseudogene) is highly conserved, with 96% sequence homology to the 16kb upstream *GBA1* locus[26]. In comparison to the *GBA1* gene, the *GBAP* gene contains shorter sequences of intronic Alu repeats and a 55 base-pair (bp) deletion in exon 9. Additional genes in the vicinity of the *GBA* and *GBAP* genes include *THBS3* (thrombospondin 3 gene), metaxin and the pseudogene of metaxin. Metaxin codes for a protein on the outer mitochondrial membrane[27]. Thrombospondin 3 has been shown in mouse models to regulate skeletal maturation[31].

1.2.2 *GBA* MUTATIONS

There are over 300 reported *GBA1* mutations and approximately 80% are missense mutations[27]. In addition, nonsense mutations, insertions, deletions, frameshift changes, splice site alterations, recombinant alleles together with mutations in *cis* have been described[27]. L444P, described in 1987, was the first reported mutation in GD and the N370S allele, was discovered the following year[25;32]. Genotype analysis, from interrogation of the International Collaborative Group Gaucher Registry (ICGG), demonstrated the presence of a N370S or L444P allele in 77% and 34% of patients respectively (n=635, type I GD, mixed ethnicity)[33]. Alfonso *et al*. (2007) interrogated data from the Spanish Gaucher registry and reported that N370S and

L444P were responsible for 50.2% and 18.4% of mutant alleles respectively[34]. Analysis of 766 patients (68% Ashkenazi or Sephardic Jews) from the Gaucher registry showed that 84% had one N370S allele, 30% had one L444P allele and that homozygosity for N370S was the most frequently identified genotype[28]. In contrast, a study from Brazil, reported that 65/139 patients had the N370S/L444P genotype (Ashkenazi-Jewish, 0.5%)[35]. In patients of Jewish ancestry, 8 mutations and 2 recombinant alleles account for the genotype in 90% of cases (84GG, IVS2+1, N370S, V394L, D409H, L444P, R463C, R496H, RecNciI and RecTL)[27;36]. In one study, these mutations were reported to identify one or both mutant alleles in 63/64 or 57/64 of patients respectively[37]. Analysis from an Italian group, all of non-Jewish descent, reported that these alleles were responsible for 92.4% of *GBA1* mutations[38]. In an Hungarian cohort, 26/27 of whom were of non-Jewish ethnicity, screening for the mutations listed above would have failed to detect 11/54 disease causing alleles[39]. Horowitz *et al.* (1993) reported that these mutant alleles, excluding the V394L mutation (not checked for), would have detected in total 88% and 77% of mutant alleles in patients of Jewish and non-Jewish descent respectively[40]. Analysis of 766 Gaucher Registry patients revealed the most common alleles to be N370S(53%), L444P(18%), 84GG(7%) and IVS2+1(2%)[28]. Amongst an Ashkenazi Jewish population, these four mutations have been reported to make up 93% of mutations[37]. Therefore, in summary, allele frequency varies with ethnicity and geographical location.

Disease causing mutations have not been described in exon 1. The majority of *GBA1* mutations are in two exons blocks (exons 5-7 and 8-11). N370S, the commonest mutated allele results from a mutation in exon 9, whereas the L444P allele is derived from mutation or a recombinant allele in exon 10 (Figure 1-3)[27].

The X-ray structure of lysosomal glucocerebrosidase has been described[41] and is composed of 3 domains with Domain III containing the catalytic site. Although, some mutations around the active site predict for a severe phenotype, generally the spatial location of the mutation does not correlate with disease severity[17].

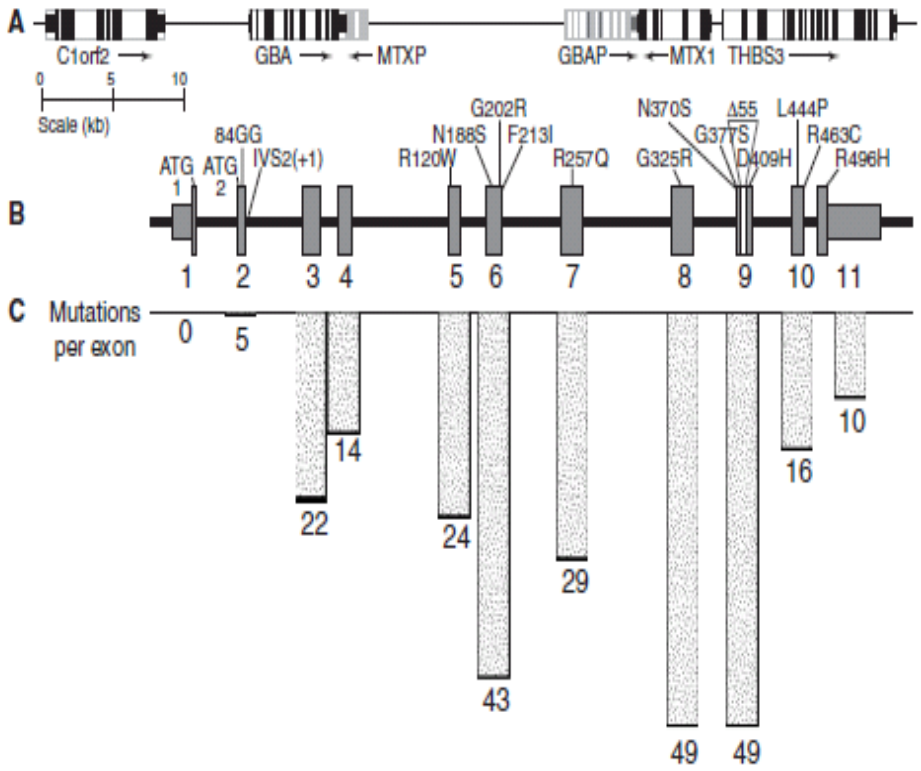


Figure 1-3. *GBA1* gene, mutations and neighbouring loci.
(A) Structure of the *GBA1* gene and neighbouring loci on chromosome 1q. *GBA1* (glucocerebrosidase), *MTXP* (metaxin pseudogene), *GBAP* (glucocerebrosidase pseudogene), *MTX1* (metaxin 1) and *THBS3* (thrombospondin 3). (B) *GBA1* gene contains 11 exons and 15 common mutations are illustrated. (C) Number of reported mutations per exon including insertions, deletions, splice site mutations and substitutions.
[Reproduced from Hruska *et al.* (2008)][27]

1.2.3 CLINICAL PRESENTATION

Clinically GD is divided into non-neuronopathic (type 1) and neuronopathic (types 2, 3) subtypes[42]. Charrow *et al*. (2000) reported type I, type II and type III disease in 94%, <1% and 5% of 1,698 individuals in an international Gaucher registry respectively[28]. The clinical phenotype of type I disease includes hepato-splenomegaly, bone marrow failure, cytopenias, skeletal disease, bleeding diathesis, liver fibrosis, fatigue, weight loss, growth retardation and gammopathy[15;24;28;43-45]. Observed skeletal disease includes osteoporosis, the Erlenmeyer flask deformity, osteopenia, avascular necrosis, bone marrow infiltration, lytic lesions, pathological fracture, osteonecrosis and osteosclerosis[28;46]. The Erlenmeyer flask deformity is the commonest skeletal anomaly (46%)[28], has a classic appearance on X-ray examination and is secondary to impaired re-modelling of the tibial metaphyseal region[46]. Patients with GD (8%), not-uncommonly, require joint replacement[28]. Pulmonary

hypertension is not infrequent in type I GD and is more prominent in asplenic individuals[47]. Parkinson's disease has been reported to be more common in individuals with type I GD[48].

Type 2 disease (early onset acute neuronopathic subtype) is characterised by oculomotor apraxia, corneal opacities, progressive neurological deficit and death at an early age (<5 years)[15]. Type 3 Gaucher disease, subacute neuronopathic subtype, presents with a mixed clinical phenotype incorporating features of both type 1 and 2 disease with a life expectancy of early adulthood (<30 years)[15]. Genotype is predictive, on the whole, for whether a patient has neuronopathic or non-neuronopathic disease. Data from the Gaucher registry reported that all patients who were carriers of a N370S mutation, homozygotic or heterozygotic (84% of 766 patients), were labelled as having type 1 disease[28]. These findings mirror those of Koprivicia *et al.* (2000) who reported genotype-phenotype correlations in a smaller cohort of 152 patients (128 type I disease; 24 type III disease)[37].

The L444P allele is the most frequent mutation found in neuronopathic subtypes[28;37;49]. Gaucher register data from 52 patients with homozygous L444P mutations revealed 75% to have a phenotype compatible with type III disease with the other 25% having clinically type I disease[28]. However, neurological assessment of 31 patients with type 1 GD, revealed abnormalities in approximately 30% of patients, including peripheral neuropathy, dementia, features of parkinsonism, abnormal eye movements and psychomotor retardation[50]. Neurological complications associated with type I GD were reported to occur frequently in a Dutch cohort of patients[51]. These findings have challenged the subdivision of GD into neuronopathic and non-neuronopathic subtypes.

Even for those with type I GD that carry the same genotype there can be marked variation in both disease severity and clinical phenotype[34;39]. Patients with homozygous N370S mutations, compared to other genotypes, have been suggested to have a higher rate of skeletal disease but less prominent visceral bulk[52]. The N370S/N370S genotype is generally associated with milder disease and presents at a later mean age[28]. Although, many children with this genotype, present with severe disease at an early age[33].

1.2.4 PATHO-PHYSIOLOGY IN TYPE I GAUCHER DISEASE

Deficiency in glucocerebrosidase leads to the accumulation of glucosylceramide and its de-acetylated form glucosylsphingosine, which is undetectable in normal tissues[42]. In addition, intermediate metabolites of sphingolipid metabolism, including those derived from the enzymatic degradation of membrane gangliosides and globosides, accumulate in the organs of those with GD[24]. The accumulation of secondary substrates, such as glucosylsphingosine, may be as important pathologically, as glucosylceramide storage, in the cellular abnormalities seen in GD.

Macrophages/monocytes are phagocytic cells and have significantly higher glucocerebrosidase activity compared to haematological cells derived from other lineages[53]. Blood glucosylceramide levels are increased in GD and have been reported to be higher in type III patients than those with type I disease[54]. Patients with GD have insufficient enzyme activity in their macrophages to catabolise membrane sphingolipids from the phagocytosis and degradation of senescent leucocytes and red blood cell membranes. This leads to intra-cellular lipid accumulation and the accumulation of Gaucher cells or foamy macrophages[55]. Cells derived from the monocytic lineage, including tissue macrophages in the liver (Kupffer cells), bone (osteoclasts), lung (alveolar macrophages), bone marrow, brain (microglial cells), spleen, lymph-nodes, gastro-intestinal tract, serous tissue, skin and urinary tract are all affected[42]. Patients with L444P/L444P, a genotype conferring neuronopathic disease, are associated with low residual glucocerebrosidase activity (<1%-13%)[56]. Speculatively, neuronopathic disease may require lower residual enzyme activity, as microglial cells are not exposed to the same substrate load as peripheral macrophages. The composition of glucosylceramide is also different. Post-mortem analysis, in a type II GD patient, demonstrated a predominance of stearic acid containing glucosylceramide in the central nervous system, in contrast to palmitic acid complexed glucosylceramide in visceral organs[57].

Gaucher cells (20-100μm) accumulate in sheets within enzyme-deficient tissue and are associated with an inflammatory infiltrate, fibrotic reaction and the deposition of extra-cellular sphingolipid[24;45]. Gaucher cells are tartrate resistant acid phosphatase (TRAP) positive, express monocyte markers and have been shown to have a phenotype of alternatively activated macrophages[58]. They are round cells, exhibit an eccentrically placed nucleus, uni-nucleate or multi-nucleate and contain large

volumes of cytoplasm with the appearance of crumpled tissue paper[59]. X-ray crystallography demonstrates that Gaucher cells accumulate lipid in membrane-bound tubular structures[60].

Hepatic pathology includes fibrosis, sheets of lipid laden Kupffer cells and extra-medullary haematopoiesis[61]. Hepatomegaly is a clinical hallmark of GD, although hepatocytes are generally spared from glucosylceramide deposition, perhaps due to excretion in the bile[62]. The incidence of gallstones has been suggested to be increased in GD[63]. Cirrhosis, liver failure, portal hypertension, hepato-pulmonary syndrome and oesophageal varices, although rare, are seen in type I GD[45;64]. Successful long-term outcomes post liver transplantation in type I GD have been reported[64]. Pathological changes in the spleen include organomegaly, Gaucher cell infiltration, infarction, extra-medullary haematopoiesis, fibrotic change and nodules that are sometimes mistaken for cancerous infiltration on radiographical examination[61]. Glucosylceramide content, in grossly enlarged visceral organs, including the liver, has been shown at post-mortem to account for less that 2% of the total weight[24]. Lymphadenopathy, is secondary to the infiltration of Gaucher cells, but little inflammatory reaction, is not uncommon[61].

Bony pathology includes lytic lesions, an accumulation of osteoclasts, abnormalities of the microcirculation, fracture, sclerosis, demineralisation, deformity, fibrotic change, a dense infiltration by Gaucher cells, infarction, necrosis, loss of trabecular connectivity and porous trabeculated cortices[24;42;61]. Type I GD is associated with an increased incidence of clonal plasma cell disorders and non-Hodgkin lymphoma[65;66]. Bone marrow failure ensues, secondary to Gaucher cell infiltration, and cytopenias are compounded by concomitant sequestration secondary to organomely[66].

Pulmonary disease is rare but alveolar macrophages are affected as demonstrated morphologically by light and electron microscopy post broncho-pulmonary lavage in a young GD patient with respiratory symptoms[67]. Infiltrates of Gaucher cells have been reported to occur in the lung interstitium and alveolar air spaces, mimicking consolidation[61]. Pulmonary involvement can lead to abnormal X-ray examination, impaired lung function tests, hypoxia and an interstitial infiltrate on high resolution computer tomography (CT) scanning[68;69]. Miller *et al.* (2003) reported that in a

cohort of 150 patients with GD, <5% had evidence of pulmonary disease[70]. In contrast, in a study reported by Kerem *et al.* (1996), approximately two thirds of individuals were shown to have abnormal lung function tests with 17% having an abnormal chest X-ray[69]. Pulmonary hypertension has been suggested to occur more commonly post-splenectomy[71] and may complicate liver pathology[64]. Echocardiography demonstrated pulmonary hypertension in 7% of 134 analysed individuals with type I disease based on the assessment of tricuspid incompetence with a gradient of greater than 30mmHg[71]. It should be noted that clinically significant pulmonary hypertension is rarely encountered in practice and both echocardiography and pulmonary function tests are associated with significant technological limitations including reproducibility. Splenectomy is associated with an increased incidence of pulmonary hypertension in non-GD individuals adding further doubt to the aetiology of these abnormalities being related to lysosomal dysfunction. Cardiac, renal and gastro-intestinal disease has rarely been reported in GD[61].

Many studies have been published regarding the plasma levels of cytokines, growth factors and chemokines in patients and are summarised in Table 1-1 below. Conflicting data has been reported regarding serum levels of pro-inflammatory cytokines including Interleukin (IL)-1, IL-2, Tumour necrosis factor-alpha (TNF-α) and IL-6[72-77]. GD is associated with an elevated incidence of gammopathy and serum levels of B-cell proliferating factors, including IL-6, IL-8 and IL-10, have been the subject of contrasting reports in the literature[72;74;76;78]. However, cytokines promoting monocyte maturation, leading to osteoclast differentiation, that are the product of activated macrophages have most consistently been reported to be elevated in the serum of those with GD. These include macrophage colony stimulating factor (M-CSF), macrophage inflammatory protein (MIP) 1α, MIP1-β and pulmonary and activation regulatory cytokine (PARC)[72;78-80]. Furthermore, there is an isolated report of raised serum levels of hepatocyte growth factor (HGF), a cytokine known to promote osteoclastogenesis[81].

Cytokine	Description	Cellular Origin	Investigator Findings
IL-6 or IL-6 mRNA	B-cell stimulation Acute Phase Response Pro or anti – inflammatory response	Macrophages T-cells	Yoshino *et al.* (2007) - 0/8 De Fost *et al.* (2006) - ↑4/24 Barak *et al.* (1999) – ↑levels* Hollak *et al.* (1997) – normal Lichtenstein *et al.* (1997) – ↑3/19 (IL-6 mRNA) Allen *et al.* (1997) - ↑levels*
IL-8	B-cell stimulation Pro-inflammatory	Macrophages Epithelial cells T-cells	Yoshino *et al.* (2007) - 0/8 Barak *et al.* (1999) – normal Hollak *et al.* (1997) – ↑93%* Lichtenstein *et al.* (1997) – ↑3/19 (IL-8 mRNA)
IL-10	B-cell proliferation and antibody secretion Anti-inflammatory	Macrophages Monocytes Lymphocytes	De Fost *et al.* (2006) - ↑17/24 Allen *et al.* (1997) - ↑levels*
TNF-α	Inflammation Acute phase response Inhibits viral replication Anti-tumour effect Chemo-attractant	Macrophages Lymphocytes	Yoshino *et al.* (2007) - 1/8 Altarescu *et al.* (2005) - ↑1/12 Barak *et al.* (1999) – ↑3/21 Hollak *et al.* (1997) – normal Lichtenstein *et al.* (1997) - ↑ 2/19 (TNF-α mRNA) Allen *et al.* (1997) - ↑levels 4/11 Michelakakis *et al.* (1996) – ↑64%
IL-1α IL-1β IL-1 Receptor Antagonist (RA)	Pro-inflammatory Pyrogen Leucocyte migration	Macrophages Monocytes Dendritic cells Fibroblasts Lymphocytes Granulocytes	Yoshino *et al.* (2007) - ↑ 1/8 De Fost *et al.* (2006) - ↑4/24 Barak *et al.* (1999) – ↑levels IL-1 β*, IL-1 RA* Hollak *et al.* (1997) – normal Lichtenstein *et al.* (1997) – ↑5/19 (IL-1 β mRNA) Allen *et al.* (1997) – IL-1β, not detectable
PARC	Chemotactic for activated T-cells and non-activated lymphocytes	Macrophages Monocytes Eosinophils	De Fost *et al.* (2006) - ↑24/24 Boot *et al.* (1997) - ↑levels in 100%*
Soluble IL-2R	Pro-inflammatory Lymphocyte stimulant Pyrogen	T-Lymphocytes	Barak *et al.* (1999) – ↑levels IL-2R*
M-CSF	Macrophage differentiation	Osteoblasts Bone marrow stromal cells	Yoshino *et al.* (2007) - ↑ 1/8 Hollak *et al.* (1997) – ↑86%*
MIP-1α	Implicated in myeloma bone disease	Macrophages	Van Breeman *et al.* (2007) - ↑mean levels
MIP-1β	Implicated in myeloma bone disease	Macrophages	Van Breeman *et al.* (2007) - ↑mean levels

Table 1-1. Summary of plasma cytokines, chemokines and growth factors elevated in GD. PARC (pulmonary and activation regulated cytokine), M-CSF (monocyte colony stimulating factor), RANK-L (receptor activator of nuclear kappa-B ligand) and MIP (macrophage inflammatory protein). *Statistically significant ($p<0.05$), compared to a control population

1.2.5 CLINICAL BIOMARKERS AND DISEASE SEVERITY SCORES

Plasma biomarkers used commonly for monitoring disease severity in GD include serum angiotensin converting enzyme (S-ACE)[82], chitotriosidase acitvity[83], ferritin[84], CCL18 (Chemokine [C-C motif] ligand 18)/PARC[80] and acid phosphatase[85]. As discussed below, these biomarkers are elevated in other macrophages diseases, in addition to GD.

TRAP, a specific acid phosphatase, expressed in osteoclasts and dendritic cells, is characterised by its lack of inhibition to tartrate. TRAP is elevated in GD[24;86] but serum levels seem not to mirror the severity of skeletal disease. Chitotriosidase activity, a macrophage-derived enzyme, correlates with clinical burden and decreases with disease-modifying therapy[83;87]. Levels may rise greater than 200-fold in severe disease, whereas levels under 1000 units/litre (normal <150 units/litre) generally indicates stable disease[15]. In addition to GD, vastly elevated activity is seen in children with Niemann Pick A or B[88]. Milder elevations in chitotriosidase activity are seen in elderly individuals[89] and in those with sarcoidosis[90]. Korolenko *et al.* (2000) argued that chitotriosidase activity is a surrogate marker of macrophage activation. The authors demonstrated an increase in enzyme production in macrophages challenged with zymosan or Triton WR-1339[91]. Boven *et al.* (2004), using in-situ hybridisation, demonstrated chitotriosidase production specifically in Gaucher cells, but not in normal macrophages[58]. Approximately 6% of the population have undetectable chitotriosidase activity due to homozygous null mutations in chromosome 1[92]. Furthermore a further third of individuals are heterozygous for a null mutation and are expected to have half the activity of those with functional genes[92].

PARC, a biomarker measurable in serum or urine, is produced by Gaucher cells[58], correlates with visceral bulk[93], parallels chitotriosidase activity[80] and can be used to monitor disease severity in those with non-functional chitotriosidase genes[94]. Although, not to the same magnitude as seen in GD, PARC is elevated in the serum of those with beta thalassaemia[95]. Niemann Pick B, a LSD associated with the accumulation of foamy macrophages and sea blue histiocytes, is associated with elevated CCL18 levels[96]. Sarcoidosis is a disorder characterised by granuloma and macrophage pathology. Chitotriosidase activity and CCL18 levels reflect disease severity and predict for clinical relapse in sarcoidosis[97]. Ferritin, a protein complexed with iron, is stored in macrophages of the reticulo-endothelial system. Serum ferritin levels are raised in GD[84] and thalassaemia[95]. In addition, high levels of serum ferritin are seen in haemophagocytic syndrome, a disorder characterised by cytopenia, fever, hepato-splenomegaly and the infiltration of organs by macrophages with enhanced phagocytic activity[98]. Stimulated or activated macrophages secrete storage ferritin in

many clinical states, including those associated with inflammation or infection. Furthermore, malignant states are associated with hyperferritinaemia and the storage of complexed ferritin in macrophages, secondary to hepcidin dysregulation and the production of pro-inflammatory cytokines[99].

S-ACE is a useful serum marker for monitoring disease severity and the response to disease-modifying therapy in GD[100]. However, S-ACE is elevated in several other disorders associated with macrophage pathology, including those of alveolar macrophages such as sarcoidosis[101]. S-ACE is expressed in many other tissues including vascular endothelium, heart, brain, adrenal cortex, leucocytes, kidney, monocytes, neurons and uterus[102]. S-ACE converts angiotensin I to angiotensin II which controls blood pressure by stimulating the adrenals to produce aldosterone. Angiotensin II has been suggested to lead to increased free radical production, decreased nitric oxide generation and the secretion of pro-inflammatory cytokines, including TNF-α plus IL-1[102]. Although data are conflicting, ACE-inhibitors have been associated with a lower incidence of cancer, including oesophageal[103]. Whether elevated S-ACE levels and enhanced angiotensin II production contributes to an elevated risk of cancer in GD is unclear.

Traditionally, the Zimran severity score (ZSS)[104], described in the era prior to enzyme replacement therapy and before the discovery of serum biomarkers, has been used to document disease load in GD. This severity index contains scoring domains for bony disease, spleen status, age at presentation, liver function abnormalities, hepatomegaly, cytopenias, splenomegaly, clinical signs of liver disease and the involvement of organs not fore-mentioned. The Zimran score is insensitive for assessing the response to ERT as prior splenectomy and osteonecrosis are not reversible variables[105]. Proposed severity scoring indexes, including the Gau-SSI[106], that address these issues and incorporate biomarker data, may prove more sensitive in monitoring clinical response.

Charrow *et al.* (2000) reported that genotype was predictive of disease severity and correlated with the mean age at presentation (N370S/N370S – 27.2yrs, N370S/? – 18.1yrs, N370S/L444P – 16.2yrs, L444P/L444P – 2.3yrs, N370S/IVS(2+1) - 6.4yrs)[28]. However, each genotype was associated with a wide range of presentation

ages. Analysis of a Spanish cohort demonstrated significant clinical heterogeneity in patients carrying a N370S allele. The authors reported that 6% of heterozygotes for a N370S allele had neurological disease with marked clinical variation in N370S/L444P patients[34]. Neither residual enzyme activity or genotype correlates well with disease severity[107]. N370S homozygotes may fail to present clinically in life whereas others have severe disease at an early age[52]. In addition, in a Canadian analysis of 24 siblings with GD, 10/24 were found to have non-concordant disease[108].

It is unclear at present which genetic and environmental factors modify disease severity in GD. Polymorphisms in the glucosylceramide synthase gene were not found to account for disease variation in individuals with homozygous N370S disease[109]. Low lysosomal glucocerebrosidase activity, due to homozygous *GBA1* mutations, leads to substrate accumulation predominantly in macrophages. In the last few decades, *GBA2*, a cell membrane associated β-glucosidase has been described[110;111]. In contrast to *GBA1*, *GBA2* is an integral membrane protein and not located to the lysosomal compartment. Ceramide formed by lysosomal glucocerebrosidase activity (*GBA1*) is rapidly converted to sphingosine and fatty acids. However, ceramide generated by *GBA2* glucocerebrosidase activity, leads to the accumulation of sphingomyelin[111]. Yildez *et al.* (2006) demonstrated the accumulation of glucosylceramide in the testes, liver and brain of *GBA2* knockout mice, but this did not lead to the development of organomegaly, neuronopathic disease or reduced life span[112]. Speculatively, *GBA2* activity may modulate GD phenotype, or prevent significant patho-physiology in cells not exposed to large amounts of substrate.

1.2.6 TREATMENT

The mainstay of GD therapy is ERT. A minority of patients are treated with substrate reduction therapy (SRT; e.g. Miglustat, N-butyl-deoxynojirimycin)[113;114] or chaperone therapy as part of a clinical trial. Historically, in the pre-ERT era, patients with severe type I or type III disease were treated therapeutically with bone marrow transplantation (BMT)[115;116]. Transplantation, however, is associated with significant morbidity and mortality. Although performed rarely in the era of ERT, splenectomy

was previously employed for painful splenic infarcts, cytopenias, diagnostic purposes and for symptom control[117]. Splenectomy leads to worsening bony disease and the development of pulmonary hypertension[118]. Additional treatment modalities include joint-replacement, analgesics, calcium supplements and bisphosphonates.

In the 1980s, glucocerebrosidase purified from human placentas, was infused into individuals with GD. Although, associated with a therapeutic effect, a large number of human placentas were required per dose and exposed the recipient to the possibility of transmittable diseases[118]. Imiglucerase, recombinant glucocerebrosidase, modified for increased macrophage uptake by the addition of terminal mannose residuals, became commercially available in 1991 (Cerezyme®; Genzyme Cooperation)[118;119]. Analysis of 1028 Gaucher registry patients treated with imiglucerase, demonstrated its efficacy in ameliorating cytopenias, visceral bulk, bony pain and episodes of bony crisis[120]. Skeletal response to enzyme therapy may be slower to improve than the haematological and visceral manifestations[121]. ERT leads to a reduction in plasma chitotriosidase activity[83]. Human recombinant enzyme, Velaglucerase[122], and a plant derived glucocerebrosidase[123] has been reported to have therapeutic effect with tolerable side-effects in early phase clinical trials. However, ERT, due to its inability to cross the blood brain barrier, is not effective in neuronopathic GD.

Miglustat (N-butyldeoxynojirimycin), an oral inhibitor of substrate synthesis in the sphingolipidoses, leads to an improvement in haematological parameters, a reduction in spleen and liver volumes, decreased chitotriosidase activity and improves bony disease in GD patients[124-126].

Mutant glucocerebrosidase, due to abnormal protein folding, is sensed by the ER where it is retained and subsequently degraded (ERAD, ER associated degradation)[16]. Mutated glucocerebrosidase leads to ER stress and is targeted to the proteasome for catabolism. Pharmacological chaperone therapy aims to stabilise or re-fold mutant enzyme, allowing trafficking from the ER to the lysosomal compartment[127]. Clinical trials are on going using chaperones, small molecules (e.g. isofagamine), which have the potential to cross the blood brain barrier. It is unclear at present whether chaperone therapy leads to a sufficient increase in residual

enzyme activity to ameliorate disease pathology in patients. In the future, as GD is a monogenetic disorder, gene therapy may become a therapeutic option[127], pending advancements in vector delivery and safety. Viral safety and transfection rates limit this approach at present.

1.2.7 PROGNOSIS

An average a life expectancy of 68 years was found in registry patients with type I GD compared to 77 years in a reference population from the United States (US)[128]. Splenectomised patients were found to have an inferior life expectancy to those with an intact spleen (72 years versus 64 years). In total, there were 102 deaths, of which 90% had received imiglucerase for a median of 5.4 years[128]. Malignancy was the cause of death in 17/63 patients with an identifiable aetiology, at a similar median age to the reference population. In the era of disease modifying therapy, where patients are treated at an early clinical stage, reducing the incidence of splenectomy and peak disease burden, life expectancy may be speculated to be higher.

1.3 GAUCHER DISEASE AND CANCER RISK

Patients with GD, compared to the general population, have been shown to have an elevated risk of both haematological and non-haematological cancer[65;78;129;130]. An early report by Lee *et al.* (1982), based on a cohort of 239 patients in the pre-ERT era, identified malignancy as the cause of death in approximately 50% of individuals[131]. Furthermore, Shiran *et al.* (1993) reported the development of cancer in 10/48 (20.8%) of their patients with GD, compared to in 35/511 (6.8%) individuals in a control population[132]. N370S homozygosity, in an American cohort, has been associated with a higher incidence of cancer (haematological and non-haematological), compared to other genotypes[52]. Splenectomised GD patients have been suggested to have an elevated cancer risk[133]. In contrast, data from an international Gaucher registry, failed to show an increased risk of cancer *per se*[130], but did conclude an increased incidence of multiple myeloma. Prior literature, reporting the development of malignancy in individuals with GD, is listed in Table 1-2. Numerically, the number of case reports describing haematological cancer in GD far outweighs those describing malignancies of non-haematological origin.

Multiple myeloma has been shown to be more prevalent in several published cohorts of patients with GD[78;130;132]. In addition, based on the number of case reports, multiple myeloma is the commonest reported cancer in GD[134-137]. Rosenbloom *et al.* (2005) analysed 2,742 patients from the international Gaucher registry, and estimated a relative risk of 5.9 for the development of multiple myeloma in those with GD[130]. Zimran *et al.* (2005), in a cohort of 500 GD patients, reported the development of multiple myeloma in 2 individuals[129]. In a combined Dutch/German cohort of 131 individuals with GD, a standardised rate ratio of 51.1 was estimated for the development of this haematological neoplasm[78]. The association of GD and multiple myeloma led to the subsequent development of consensus recommendations regarding the frequency of immunoglobulin analysis in those with GD[66].

Landgren *et al.* (2007) failed to find an increased risk of multiple myeloma, in the analysis of 1,525 adult male United States (US) veterans with GD[65]. However, criticisms of their study include its retrospective nature, the young median age of their cohort (~50yrs) and the use of non-specific coding systems to identify patients with GD. In addition, severely affected GD patients are unlikely to enrol for military service, skewing the affected population towards a milder phenotype. In the report, by Rosenbloom *et al.* (2005), 9/10 GD patients with myeloma were older than 60 years of age[130]. Interestingly, the literature is devoid of reports describing myelomatous disease in individuals with type III GD, who would typically present with severe disease at an early age, suggesting additional aetiological factors.

GD is associated with an elevated risk of polyclonal[43;81] and benign monoclonal gammopathy[138;139]. The development of malignant gammopathy from either of these two conditions, as a continuum of disease, is discussed in more detail below. AL amyloidosis, a less common malignancy of plasma cells, has been reported to occur more frequently than expected in patients with GD, based on the number of case reports[140-144]. Primary AL amyloidosis is reported to have an age-adjusted incidence of approximately 5-13 per million patient-years in the US[145]. Non-Hodgkin Lymphoma (NHL), an umbrella term encompassing both ‘low’ and ‘high grade’ lymphoma, was found to be more common in a cohort of patients with GD, compared to that expected in the general population[65]. Excluding chronic

lymphocytic leukaemia (CLL), there are only isolated case reports of 'low grade' lymphoma, including mucosal associated lymphoid tissue lymphoma[146] and splenic marginal zone lymphoma in individuals with GD[147]. Several case descriptions of common haematological malignancies including Hodgkin disease, chronic lymphocytic leukaemia and diffuse large B-cell lymphoma (DLBCL) exist in the GD literature (Table 1-2).

Non-haematological cancer including melanoma, pancreatic cancer and hepato-cellular carcinoma, have been suggested to be more common in those with GD[65;78]. Lo *et al*. (2010) reported that 9/403 of their patients, with type I GD, developed two or more malignancies, giving an incidence of 2.2%[133]. However, this was less than the reported incidence of sequential cancers (8%) in the general US population, quoted by the authors. Speculatively, these patients may have co-inherited additional cancer genes. Although, GD is a panethnic disorder, 68% of patients, based on registry data, are of Jewish descent[28]. GD is commoner in Ashkenazi Jews and this sect has a higher carriage rate than the general population of certain cancer genes, including *BRCA1* and *BRCA2* mutations. These mutations confer an elevated risk of breast and gynaecological cancer[148]. Despite this, these cancers have not been reported to be more prevalent in patients with GD. It could be hypothesised that the elevated risk of malignancy in GD may be due to gene-linkage and the co-inheritance of proto-oncogenes adjacent to the *GBA1* locus at 1q21[49]. Abnormalities at 1q21-23 are associated with early onset hepatocellular carcinoma [149]. In addition, chromosome 1 abnormalities have been reported in the plasma cell clones of approximately 45% of patients with multiple myeloma. Several genes have been reported to be up-regulated in the region of chromosome 1q22-25 in myeloma patients who have acquired gains in chromosomal material within this region[150].

In support of disease burden not being causal for malignancy, several patients experienced an episode of cancer that pre-dated their diagnosis of type I GD[136;151]. This includes a 57-year-old female who was treated with chemotherapy and an autograft for multiple myeloma. She was diagnosed with GD on a 3 month staging marrow, post-transplant, for investigation of progressive thrombocytopenia. GD was confirmed by deoxyribonucleic acid (DNA) analysis[136]. There are no reports in the literature of monoclonal gammopathy of undetermined significance (MGUS),

multiple myeloma or an increased incidence of other malignancies in paediatric patients, including those presenting with severe disease in the first few years of life. This suggests that additional factors, environmental or age-related, may be required in addition to homozygous *GBA1* mutations to inflict an elevated risk of carcinogenesis. The length of exposure to GD related pathology including substrate accumulation may be important aetiologically. Fabry disease, an X-linked disorder resulting in to a deficiency in alpha-galactosidase-A activity, has only rarely been reported to be associated with malignancy, including renal cell carcinoma[152;153] and leukaemia[154]. Therefore, lysosomal dysfunction *per se*, seems not to be sufficient to confer an elevated risk of cancer.

CANCER	Literature - Case Reports	Literature - Case Series
Haematological Cancers		de Fost *et al.* 2006[78] Shiran *et al.* 1993[132]
Non-Haematological Cancer		Taddei *et al.* 2009[52]
Multiple Myeloma	Pinkhas *et al.* 1965[155], Benjamin *et al.* 1979[156], Ruestow *et al.* 1980[157], Harder *et al.* 2000[158], Cheung *et al.* 2007[136], Gal *et al.* 1988[134], Garfinkel *et al.* 1982[137], Brady *et al.* 1997[159], Shvidel *et al.* 1995[160], Machaczka *et al.* 2009[161]	de Fost *et al.* 2006[78] Rosenbloom *et al* 2005[130] Zimran *et al* .2005[129] Taddei *et al.* 2009[52] Shiran *et al.* 1993[132] Lee *et al.* 1982[131] Shoenfeld *et al.* 1982[162]
Multiple Plasmacytoma	Garfinkel *et al.* 1985[163]	
Hodgkin Lymphoma	Cho *et al.* 1976[164], Bruckstein *et al.* 1980[165], Sharer *et al.* 1976[166], Goodman *et al.* 1988[167], Burstein *et al.* 1980[165]	
Chronic Lymphocytic Leukaemia	Mark *et al.* 1982[168], Fox *et al.* 1984[169], Kaufman *et al.* 1986[170], Marsh *et al.* 1993[171], Chang-Lo *et al.* 1975[172], Paulson *et al.* 1989[173]	
'High Grade' B-cell Lymphoma	Carreiro *et al.* 2008[174], Perales *et al.* 1998[175], Shvidel *et al.* 2007[176], Brody *et al.* 2006[177], Manz 2001[178],	
Peripheral T-cell Lymphoma	Sanchez *et al.* 2005[179]	
'Low grade' lymphoma:- **(1) Splenic-Marginal Zone Lymphoma** **(2) Maltoma**	Bertram *et al.* 2003[147] Taddei *et al.* 2009[52]	
AL-Amyloidosis	Dikman *et al.* 1978[140], Elstein *et al.* 2003[141], Hanash *et al.* 1978[142], Kaloterakis *et al.* 1999[143], Hrebicek *et al.* 1996[144]	
Chronic Myelomonocytic leukaemia	Krishnan *et al.* 2003[180]	
Acute leukaemia	Castelli *et al.* 2006[181] (ALL), Krause *et al.* 1979[182] (AML), Burstein *et al.* 1985[183] (ALL), Corbett *et al.* 1987[184] (AML)	
Chronic Myeloid Leukaemia	Shinar *et al.* 1982[185], Petrides *et al.* 1998[186]	
Hepatocellular Carcinoma	Breiden-Langen *et al.* 1991[187], Erjavec *et al.* 1999[188], Xu *et al.* 2005[189]	de Fost 2006[78]
Primary bone cancer	Kenan *et al.* 1996[190] (Osteoblastoma), Bohm *et al.* 2001 (Leiomyosarcoma)	
Prostate Cancer	Lo *et al.* 2010[133]	
Pancreatic Cancer		Landgren *et al.* 2007[65]
Lung Cancer	Tsung *et al.* 1977[191], Leone *et al.* 2008[192]	
Melanoma		Landgren *et al.* 2007[65]
Breast Cancer	Gal *et al.* 1988[134]	
Malignant Epitheloid Haemangioendothelioma	Pins *et al.* 1995[193]	
Glioblastoma Multiforme	Lyons *et al.* 1982[194]	
Dysgerminoma	Kojiro *et al.* 1983[195]	

Table 1-2. Publications describing episodes of cancer in patients with Gaucher disease.

1.3.1 PLASMA CELL NEOPLASMS

Plasma cells, first described by Waldeyer in 1875[196], are terminally differentiated B-lymphocytes that have class switched and secrete immunoglobulin of a specific isotype. Plasma cells have undergone clonal re-arrangement of their immunoglobulin genes together with somatic hyper-mutation in their variable regions. Plasma cells are absent from the peripheral blood in health, comprise 1-5% of all nucleated bone marrow cells and are present in lymphoid tissue[197]. Morphological features on light microscopy (Giemsa staining) include cytoplasmic basophilia, an eccentrically placed nucleus, clumped chromatin and a prominent peri-nuclear golgi zone[197]. Plasma cells are typically uni-nucleate although multi-nucleate forms may be seen in normal individuals. Typically plasma cells have a diameter of between 15-20μm and form small clusters, either in the interstium or around capillaries, in normal bone marrow[197]. Characteristic electron microscopic appearances include a double membraned nucleus, an abundant Golgi system and cytoplasm packed with ER and scattered mitochondria[196]. Non-malignant plasma cells express cell surface CD[cluster of differentiation]19, CD38 and CD138 but lack CD56 together with light chain restriction on immuno-phenotyping[198].

MGUS is a clonal disorder of plasma cells that is associated with either a serum or urinary paraprotein in the absence of end organ impairment. MGUS is associated with a plasma cell infiltrate of less than 10% and a paraprotein level of <30g/l[199]**.** Furthermore, deletions of 13q, hyperdiploidy and translocations involving the IgH [immunoglobulin-heavy chain] locus (14q32) are reported in about 50%, 40-50% and 40% of patients with benign gammopathy respectively[198]. Clonal plasma cells are CD19-ve, CD38+ve, CD138+ve, light chain restricted with variable CD56 expression on immunophenotyping[198]. The incidence of MGUS increases with age[200] and according to a Minnesota based study of 21,462 people, is 3.2% in those >50 years old and 7.5% in those >85 years old[201]. The frequency of MGUS in other published cohorts is shown in Table 1-3 and illustrates geographical differences in incidence. Monoclonal gammopathy is commoner in blacks than whites[202]. The incidence of MGUS in Ashkenazi Jews has not been reported in the literature. Population studies have demonstrated that around 1% per year of patients with MGUS progress to a haematological malignancy[203]. Kyle *et al*. (2002) showed that

pre-existing MGUS was most likely to transform to multiple myeloma (Relative risk [RR], 25.0) with lymphoma (RR 2.4), amyloidosis (RR 8.4), chronic lymphocytic leukaemia (RR 0.9) and solitary plasmacytoma (RR 8.5) being subsequently diagnosed in a minority of individuals[203].

Country	Age	Number of individuals	Incidence	Authorship
America	≥70	816	3.6%	Cohen *et al.*[204]
Greece	>50	1564	3.9%	Anagnostopoulos *et al.*[205]
France	≥50	17,968	1.7%	Saleun *et al.*[200]
Ghana	≥50	917	5.9%	Landgren *et al.*[206]

Table 1-3. Frequency of MGUS in different geographical populations.

Approximately 84% of 1,384 individuals with MGUS in an American based cohort had a presenting paraprotein of under 20g/l[203]. In this study Ig(Immunoglobulin)G, IgA, IgM and bi-clonal paraproteins were found in 70%, 12%, 15% and 3% of individuals respecitively[203]. These findings are in keeping with the isotype frequency reported in a Greek population[205]. Risk factors for progression from MGUS to multiple myeloma include immunoglobulin isotype (IgA>IgG)[203], an abnormal serum light chain ratio[207], a high paraprotein level[199], increased bone marrow angiogenesis[208] and the percent of infiltrating plasma cells on staging bone marrow examination[209]. Age, sex and specific cytogenetics are not thought to be risk factors for malignant transformation[199]. Cesana *et al.* (2002) suggested the Bence-Jones proteinuria, a high erythrocyte sedimentation rate (ESR) and immunoparesis to confer a higher rate of malignant transformation[209].

Multiple Myeloma is a malignant haematological disorder secondary to the accumulation of clonal plasma cells with rearranged heavy and light chain immunoglobulin genes. Elevated plasma cell numbers, dysplastic morphology, abnormal clustering and multi-nuclearity are features found on bone marrow examination that are suggestive of malignant disease[198]. Additionally, the presence of plasmablasts (high nuclear: cytoplasmic ratio, increased cell size, dispersed chromatin and visible nucleoli), mitotic figures and plasma cells with flamed shaped eosinophilic cytoplasm (flame-cells), raise suspicion of neoplastic disease. Malignant

plasma cells may accumulate immunoglobulin in cytoplasmic vacuoles (Mott cells), the cisternae of distended ER (Russell bodies) or impinge on the nucleus, secondary to cytoplasmic accumulation, giving rise to Dutcher bodies[196;198;210]. Immunophenotyping normally reveals a population of malignant plasma cells that are CD19-ve, cytoplasmic Ig+ve, CD56+ve (67-79%), CD38+ve, CD138+ve with variable expression of cyclin D1, CD20, CD52, CD10 and CD117[198].

Data from the office of national statistics reported an incidence of multiple myeloma of 3.2 per 50,000 individuals with male predominance (**http://info.cancerresearchuk.org/ cancerstats /types /multiplemyeloma/incidence**). Diagnostic criteria for symptomatic myeloma, as stipulated by the international myeloma working group (IMWG), are a plasma cell infiltrate of >10% and the presence of related end organ impairment (ROTI) including renal failure, skeletal disease (lytic lesions, vertebral collapse or osteoporosis), hypercalcaemia, anaemia, amyloidosis, recurrent bacterial infection or hyperviscosity[199;211]. Based on a study from the Mayo clinic, 43% of patients had a paraprotein less than 30g/l at diagnosis, but 96% of patients had an infiltrate of >10% plasma cells on bone marrow examination[212]. Therefore, complete and careful interpretation of staging investigations is mandatory at presentation. Malignant plasma cells are capable of metastasis and can lead to end organ impairment by either direct infiltration or by paraneoplastic effect. Approximately 3% of individuals have non-secretory myeloma (no urine or serum M-band) with a further 20% being categorised as light chain myeloma[212]. Below describes the frequency of specific serum monoclonal bands from this study[212]:-
[IgGκ (34%), IgGλ (18%), IgAκ (13%), IgAλ (8%), IgMκ (0.3%), IgMλ (0.2%), IgDκ (1%), IgDλ (1%), Free κ only (9%), Free λ only (7%), Bi-clonal (2%) and Negative (7%)]

IgG Myeloma is commoner than those sub-typed as IgA with these two isotypes being expressed in 73% of cases[212]**.** IgD and IgE Myeloma are rare, suggested to confer a poor prognosis and warrant case reports within the literature[213;214]**.**

Current first-line therapy in patients with symptomatic myeloma from the Royal Free Hospital involves a combination of an alkylating agent, steroid and an immuno-modulatory agent. For younger patients eligible for high dose therapy with stem cell rescue, 'CDT' chemotherapy (cyclophosphamide, dexamathasone and thalidomide) is instigated. 'CDT' has been shown to be superior to 'C-VAD' (cyclophosphamide, vincristine, adriamycin and dexamethasone) as an induction regime (Morgan *et al.* 2007; Myeloma IX trial; ASH abstract). MPT (melphalan, prednisolone and thalidomide) has been shown to induce superior remission rates and event-free survival, compared to MP chemotherapy in elderly patients unsuitable for bone marrow transplantation[215]. Bortezomib, a proteasome inhibitor, has potent anti-myeloma activity in clinical trials in both newly diagnosed patients and those with relapsed disease[216;217]. Lenalidomide, a second generation immuno-modulatory agent, in combination with dexamethasone leads to superior response rates and overall survival compared to dexamethasone therapy alone[218]. In addition, lenalidomide/dexamethasone, has been suggested an effective treatment strategy in relapse[219]. In the UK, the National Institute of Clinical Excellence (NICE) has approved bortezomib in first relapse and a lenalidomide containing regimen as third-line therapy. In the era of novel therapies (lenalidomide, bortezomib *etc.*), phase III trials are being conducted to re-assess the role of autografting as a consolidative procedure post first-line therapy[220]. Bisphosphonates, inhibitors of osteoclastogenesis, are mandatory adjuvant therapy in the treatment of myeloma. Reported in abstract form, zoledronic acid has been shown to be superior to oral clodronate in the Myeloma IX trial and imparts a survival advantage (Morgan *et al.* 2010; European Haematology Association, Abstract book).

Prognosis may be predicted from several published scoring systems including the Durie-Salmon staging system, International Staging System (ISS) and from lists of adverse risk factors extrapolated from population studies, such as that reported by the Mayo clinic[211;221]. The ISS, based on presenting serum albumin and β_2-microglobulin level, categorises three risk groups. Stage I disease is defined as a β_2-microglobulin <3.5mg/l with an albumin ≥ 35g/l. Those with a β_2-microglobulin≥ 5.5mg/l having stage III disease and stage II disease includes individuals that do not meet criteria for either of these two risk groups. Survival in a cohort of over 10,000 newly diagnosed myeloma patients using the ISS predicted survival (stage I 62 months, stage II 44

months and stage III 29 months)[221] and is recommended by the IMWG for staging in the United Kingdom. In a cohort of patients >70 years old, stage I and II disease, predicted for a poorer survival than that seen in younger individuals, although, the prognosis in stage III disease was identical[222]. Based on Mayo clinic data, monosomy 13, hypodiploidy, translocation (4;14), translocation (14;16), 17p- (p53) and a plasma cell labelling index ≥3% have been suggested as adverse predictors of outcome[223]. Additionally, other investigators, have reported, a raised lactate dehydrogenase (LDH), the percentage of bone marrow plasma cells, the degree of anaemia, the number of positive lesions by positron emission tomography (PET), elevated serum M-CSF, hypercalcaemia, progression to plasma cell leukaemia, plasmablastic morphology and a high C-reactive protein to predict for inferior survival[222;224-226].

Asymptomatic myeloma is defined by the IMWG as a paraprotein >30g/l and/or a plasma cell infiltrate on bone marrow staging of >10%, in the absence of ROTI[211]. Kyle *et al.* (2007) described the rate of progression to malignant disease in 276 individuals as 10% per year for the first 5 years, 3% per year for the next 5 years and then 1% per year thereafter[227]. Risk factors, reviewed by Bladé *et al.* (2010), for progression to symptomatic myeloma include the percentage of infiltrating plasma cells, paraprotein level, the presence of circulating plasma cells, a high plasma cell labelling index, abnormal signal intensity on magnetic resonance imaging (MRI) examination, elevated serum light chains and an abnormal immunophenotype, including CD56 expression[228]. Patients with asymptomatic myeloma are carefully observed and not treated until the development of ROTI.

Primary AL-amyloidosis is caused by a clonal population of plasma cells that secrete an amyloidogenic light chain, usually lambda, capable of forming beta-pleated sheets that stain positive with Congo red[229]. Organ dysfunction correlates with amyloid fibril deposition and is usually secondary to a small population of bone marrow plasma cells with similar cytogenetics and immunophenotyping to those with MGUS or symptomatic myeloma[198]. Deposited organ amyloid contains non-fibrillar macromolecules including serum-amyloid protein (SAP)[230]. Presentation is heterogeneous but includes renal failure, proteinuria, cardiac dysfunction, liver impairment, hepato-splenomegaly, neuropathy, low level paraproteinaemia, elevated

serum light chains, macroglossia, abnormal bleeding/bruising and gastrointestinal disturbance[229]. Disease bulk can be serially assessed by a combination of routine blood tests, serum light chain analysis, echocardiography and serum amyloid protein (SAP) scintigraphy[229;231;232]. Prognosis is inferior to those with multiple myeloma with an average life expectancy of 3.3 years[230]. Chemotherapy regimens are similar to those used in symptomatic myeloma. Treatment duration may be attenuated in some individuals as responses in many are obtained early due to the presence of low tumour burden[230]. The upfront role of autografting in AL-amyloidosis is currently being investigated.

1.3.2 GAUCHER DISEASE AND GAMMOPATHY

Polyclonal gammopathy is defined as an isolated or general increase in serum immunoglobulin levels without a detectable urinary or serum paraprotein by gel electrophoresis, densitometry and immunofixation[199]. This state is not associated with clonally arranged plasma cells or end-organ tissue impairment. For several decades the incidence of polyclonal gammopathy and paraproteinaemia has been known to be increased in patients with GD[138;139;233]. Publications pertaining to the incidence of gammopathy in GD are documented in Table 1-4 below. The incidence of polyclonal gammopathy, has been suggested to be higher in paediatric GD populations (81-91%)[234;235] and this may reflect a cohort of patients presenting with severe early-onset disease. This supports disease severity being a risk factor for polyclonal gammopathy. In a report by Khalifa *et al.* (2010), which contained 12/16 children with more aggressive type III disease, polyclonal increases in IgM were seen in approximately 80% of patients[235]. The incidence of polyclonal gammopathy in adult cohorts has been reported in up to 60% of individuals, with IgG being the commonest elevated immunoglobulin[43;74;139;162].

Author	Patient Number	Age Years	Incidence of Polyclonal Gammopathy	Incidence of a Paraprotein	Incidence of Malignant Gammopathy
Pratt *et al.* 1968[138]	16	9-70	6/16 (37.5%) 6/16↑IgG (37.5%) 5/16↑IgM (31%) 4/16↑IgA (25%)	4/16 (25%) All IgGκ	0/16
Shoenfeld *et al.* 1982[162]	25	24-78	15/25 (60%)	2/25 (8%)	2/25 (8%) 2 Non-secretory myeloma
Marti et al.1988[139]	23 (type I)	Mean 42	↑IgG-15/23 (65%) Diffuse ↑Ig 10/23 Pre-ERT era	8/23 (35%) Oligo+Monoclonal Pre-ERT era	NR
Allen et al. 1997[74]	22 (type I)	23-65	14/22 (65%) ↑IgG (commonest; mixed ERT status)	2/22 (9%), monoclonal	1/22 (4.5%) Myeloma IgA
Brautbar *et al.* 2004[43]	149	17-80	93/149 (62.4%) ↑IgG (commonest; 40/149, 26.8) ↑IgM 33/149 (22%) Untreated	1% Adult patients Treated/Untreated	NR
Wine *et al.* 2007[234]	23 (type I)	Mean 8.5	21/23 (91%) (Pre-ERT) >1 Ig isotype elevated (15/23)	NR	NR
de Fost *et al.* 2008[81]	63 Type I	25-83	26/63 (41%)	13/63 (21%) IgGκ commonest 3 Bi-clonal 1 Tri-clonal	3/63 (4.8%) 1 Myeloma 2 Amyloidosis
Grosbois *et al.* 2009[236]	107 (105 type I, 2 type III)	46-78	NR	17/107 (16%) IgG:- 13 cases κ>λ 1 Bi-clonal 1 Tri-clonal 5 Asplenic	3/17 cases 1 Myeloma 2 Lymphoma
Khalifa *et al.* 2010[235]	16 (4 type I, 12 type III)	6 months – 16 years	Polyclonal gammopathy 8/16 (50%); IgM elevated in 87.5%	NR	NR

Table 1-4. Prior cases series relating to gammopathy in Gaucher disease.
NR, not reported.

Brautbar *et al.* (2004), demonstrated a reduction in polyclonal immunoglobulin levels (IgG, IgM and IgA), with the introduction of ERT[43]. In a paediatric

population, disease-modifying therapy was shown to decrease IgA and IgM levels[234], suggesting disease severity as a contributing factor to the development of gammopathy. However, in an Israeli based study, polyclonal gammopathy was not associated with markers of severity, including the Zimran score, splenectomy status, genotype or the presence of bony disease[43]. However, no correlation was made with biomarkers, such as serum chitotriosidase activity[83], that reflect disease bulk and mirror the clinical response to ERT. In a cohort of children with GD (type I/type III), age, platelet count, haemoglobin, gender and type III disease were not predictive of gammopathy[235]. Current literature is insufficient to answer whether spleen volume, liver volume, biomarker activity (S-ACE, acid-phosphatase, chitotriosidase activity) or bone marrow macrophage burden are risk factors for polyclonal gammopathy in GD.

The incidence of paraproteinaemia (8-35%), reported in Table 1-4, as discussed above, is higher than expected in the general population[200;201]. Advanced age has been shown to be a risk factor for the development of monoclonal gammopathy in GD[81;236]. This may explain why Brautbar *et al.* (2004) reported a low incidence of paraproteinaemia (1%) in a comparatively younger cohort of ERT treated patients (mean age 42 years)[43]. In an untreated cohort, reported by Marti *et al.* (1988), a monoclonal band was found in 35% of individuals of a similar mean age[139]. Exposure time (diagnosis to treatment), prior splenectomy, Zimran severity score and baseline chitotriosidase activity did not predict for the development of MGUS in one study[81]. Serum IL-6 levels were found to be higher in GD patients with a monoclonal band than those without[74]. IL-6 is a plasma cell proliferating cytokine. MGUS is not associated with ROTI, but IgMκ cryoglobinaemia, leading to neuropathy, has been previously described in an elderly patient with GD[237].

The incidence of GD is approximately 1:59,000[18] and that of multiple myeloma, in a Minnesota based study, is 1:25,000[238]. In addition, many patients with GD, fail to present clinically in life, due to variable disease severity[52]. Therefore, the number of observed cases (Table 1-2) is greater than expected, based on probability alone. There are several case reports of AL-amyloidosis in patients with GD[81;140-144] and has been suggested to have an incidence of approximately 1:100,000 in the US general population[145]. Furthermore the increased frequency of MGUS and polyclonal

gammopathy in GD supports an increased prevalence of plasma cell disorders in this disorder.

IgG is the commonest isotype secreted in non-GD myeloma[212]. In patients with GD, reports include IgG[81;132;158;161;236], IgA[74;132;157;159;160], light chain[136] and non-secretory IgD myeloma[156]. Homozygous N370S disease has been suggested, compared to other genotypes, to be an additional risk factor for the development of multiple myeloma[52]. Those with N370S/N370S disease, compared to the general population, were reported to have an 25-times higher life time risk of developing multiple myeloma[52]. The authors also reported that within their cohort of homozygous N370S individuals, prior splenectomy was a risk factor for the subsequent development of multiple myeloma. This genotype is associated with comparatively milder disease, although rarely individuals with N370S/NS70S, have severe disease[52]. Pratt *et al.* (1968) reported that hepato-splenomegaly was found in all GD patients with either monoclonal or polyclonal gammopathy[138]. However, Cheung *et al.* (2007), described the incidental diagnosis of GD in a 57 year old lady with multiple myeloma, post autograft[136]. Evidence is currently insufficient to conclude that disease severity is a risk factor for the subsequent development of a plasma cell neoplasm.

In a report, by a French based group, 6/17 patients with a monoclonal band experienced either a reduction in the paraprotein level or its disappearance with ERT[236]. This is in keeping with the conclusions of some investigators[239] but contrasts to those of Brautbar *et al.* (2004)[43]. Another report, by de Fost *et al*. (2008), suggested that ERT reduced or stabilised the level of paraproteinaemia in GD[81]. Currently, a diagnosis of MGUS is not a clinical indication for ERT[66]. In addition, it is unknown, whether ERT prevents progression to malignant gammopathy. Furthermore, is a diagnosis of multiple myeloma, in an otherwise asymptomatic Gaucher patient, an indication for ERT? Do patients with benign gammopathy and GD, progress to multiple myeloma, at the same rate as those without GD? Collaborative meta-analysis is required, via an international registry, to clarify these points.

In addition, there is insufficient data to recommend specific chemotherapy regimens for GD patients with multiple myeloma. Prior reports of treatment with MP have led

to the development of severe cytopenia[81;136;161]. Concomitant high-dose ERT may reduce Gaucher cell burden, improve the severity of cytopenia and improve haematological recovery post chemotherapy. In one GD patient with myeloma, myelodysplasia followed by leukaemia transformation developed post-treatment with MP chemotherapy[161]. Immunomodulatory agents, including thalidomide and lenalidomide, can lead to profound cytopenias[240;241] and may be poorly tolerated in GD. In addition, Machaczka *et al.* (2009), postulated that bortezomib, may also lead to unacceptable side effects in GD patients[161]. GD causes proteasomal dysfunction[16] and the use of bortezomib may compound GD cellular pathology further. Although, there are reports in the literature, of both favourable[81] and unfavourable outcomes[161] to chemotherapy, no firm conclusion can be drawn on treatment outcome due to the low number of reported cases.

1.3.3 GAUCHER DISEASE - AETIOLOGY OF PLASMA CELL DISORDERS

Multiple myeloma and GD have overlap in their clinical presentation including bone marrow infiltration, cytopenias, and skeletal disease (lytic lesions, pathological fracture and osteoporosis)[24;242;243]. Non-GD and GD individuals with MGUS progress to malignant disease[81;244]. Patients with GD, as discussed above, have a higher incidence of polyclonal gammopathy, MGUS and multiple myeloma[43;78;81]. Gammopathy in GD therefore represents a spectrum of disease and pathological disturbances within the bone marrow microenvironment, as suggested in non-GD patients with MGUS, maybe fundamental to malignant transformation. In addition, there are several biological features common to both disorders, which may be speculated to contribute to the development of clonal plasma cell disorders. GD is predominantly a disorder of the monocytic lineage, including macrophages and osteoclasts. An accumulation of lipid laden macrophages within the bone marrow[131;245;246], elevated serum biomarkers of macrophage activity[79;247], radiological evidence of osteoclast over-activity[242;248], elevated serum biomarkers of bone resorption[249;250] and raised blood levels of cytokines promoting osteoclastogenesis[72;251] have been described in both GD and multiple myeloma. Both macrophages and osteoclasts, based on *in vitro* co-culture models, have been shown to promote plasma cell survival[245;252]. Furthermore elevation in the serum levels of

plasma cell proliferating cytokines including IL-6, a macrophage manufactured cytokine, have been reported as elevated in individuals with either myeloma or GD[74;253].

Numerical and functional abnormalities in lymphoid subsets[4;139;254;255] have been reported in individuals with either GD or multiple myeloma. Patients with GD, as discussed above, have an elevated risk of several non-haematological malignancies, including hepatocellular carcinoma[65;78;133]. These observations suggest a more global defect, extending beyond the bone marrow microenvironment, that renders an elevated cancer risk in those with GD. Impaired tumour surveillance, a concept described in patients with Human immunodeficiency syndrome (HIV) infection[256] due to depressed cellular immunity, has not been fully explored in GD.

The following theories for the development of multiple myeloma in GD are explored below, although not mutually exclusive:-

1. Disturbances of the bone marrow microenvironment
2. Chronic inflammation
3. Chronic antigen presentation
4. Impaired tumour surveillance
5. *GBA1* mutations

1.3.4 BONE MARROW MICROENVIRONMENT

The bone marrow microenvironment encompasses stromal cells, osteoblasts, osteoclasts, plasma cells, macrophages, haematopoietic cells/progenitors, the microcirculation and connective tissue including bone[257]. Over the last 2 decades many abnormalities of the bone marrow microenvironment have been described favouring plasma cell growth, expansion, malignant transformation and chemo-resistance[245;252;258-261]. Furthermore many of these abnormalities are also seen in individuals with GD, including elevated macrophage and osteoclasts numbers, suggesting potential common aetiology for the development of a plasma cell neoplasm[61;245;262].

1.3.4.1 MACROPHAGES

Macrophages are derived from the monocytic lineage[263] and these phagocytic cells have important roles in innate immunity, antigen presentation, inflammation, angiogenesis, coagulation, the breakdown of apoptotic cells, the destruction of microbes, tumour surveillance and many metabolic pathways including iron recycling[263;264]. In normal bone marrow, macrophages are large cells with voluminous cytoplasm, small nuclei and are often surrounded by plasma cells, lymphocytes or maturing erythroid cells. Their cytoplasm may be vacuolated, ruffled and may contain phagocytosed debris[197]. Monocytic precursors are driven into macrophage differentiation by colony stimulating factor-1 (CSF-1), IL-3, M-CSF and GM-CSF[265].

Tissue macrophages harbour a cocktail of signalling membrane molecules including Ig Fc receptors (FcRγR1 [CD64], FcRγRIII [CD16]), CD4, complement receptors (CR1, CR3 [CD11b/CD18]), the mannose receptor (CD206), toll like receptors (TLR), scavenger receptors, hormone receptors, chemokine receptors, lipoprotein receptors, growth hormone receptors (M-CSF, GM-CSF), fibronectin receptors and HLA proteins (class I and II)[263]. Scavenger receptors recognise low-density lipoproteins, lipopolysaccharide (LPS), dead cells and microbes[264]. LPS, peptidoglycan, bacterial DNA, RNA and several other microbial components comprise the ligands recognised by TLRs[263]. Opsonised molecules including those derived from tumour cells or microbes activate macrophages via their Fc receptors. This can lead to a host of responses including antibody mediated cell death, engulfment, enzyme secretion and the production of toxic nitrogen and oxygen species. Complement receptors mediate macrophage chemotaxis (e.g.C5a) and the recognition of non-self by the binding of complement components (e.g. C3b)[263]. The addition of mannose residues to ERT has facilitated its uptake by macrophages in GD[118].

Macrophages secrete a rheostat of cytokines, chemokines and growth factors which modulate tumour cell growth, chemoresistance, neoangiogenesis, chemotaxis and the recruitment of T-cells[266]. Macrophages can be differentiated via external stimuli into either classically activated type I macrophages (M1) or alternatively activated type II macrophages (M2)[267]. Type I macrophages are pro-inflammatory, anti-microbial,

tumourcidal and are characterised by the secretion of INFγ and TNF-α. In contrast, M2 macrophages, promote carcinogenesis[267;268], regulate tolerance, mediate an anti-inflammatory response, facilitate angiogenesis, express low levels of HLA-class II molecules and secrete IL-1RA, IL-4 and IL10[267;269]. A higher burden of macrophage infiltration has been associated with a poorer prognosis in those with glioblastoma[270]. M1 and M2 macrophages differ in their expression of membrane receptors (e.g. scavenger, Fcγ-R, CD14/64), cytokines, cytokine receptors, chemokines, chemokine receptors and effector molecules[267]. Tumour cells produce a cocktail of inflammatory cytokines including extracellular matrix components, IL-10, chemokines (CCL2, 4, 17 and 22) and CSF-1 that can recruit and polarise macrophages into a M2 phenotype[269].

Macrophages are more abundant in the bone marrow trephines of patients with myeloma than in healthy controls[245]. Zheng *et al.* (2009), using cell culture models, demonstrated that macrophages in a contact-dependent manner, up-regulated anti-apoptotic proteins, and prevented chemotherapy-induced death in co-cultured plasma cells. Furthermore, macrophages which were conditioned with the culture medium of myeloma cells afforded greater protection than unconditioned macrophages[245]. Higher numbers of infiltrating tumour-associated macrophages (TAMs) are associated with tumour invasion, lymph node metastasis and advanced disease in colorectal cancer[271]. Poor prognosis is conferred by the presence of large infiltrates of TAMs in other cancers, including sino-nasal melanoma,[272] and predicts for an inferior outcome to hormonal therapy in prostate cancer[273]. Macrophages are an important component of the malignant microenvironment and investigators are currently exploring the use of antibodies against CCL2 (promotes M2 polarisation and the recruitment of TAMs) in early phase clinical trials[267]. In addition, macrophages secrete a cocktail of cytokines promoting osteoclastogenesis (see below). Osteoclasts have been shown *in vitro* to support myeloma cell growth and survival[274].

GD, as discussed above, is a disorder characterised by the accumulation of foamy macrophages or Gaucher cells. Boven *et al.* (2004) demonstrated that Gaucher cells expressed common macrophage markers including CD68, CD14, HLA (Human leucocyte antigen)-class II and have a phenotype of M2 macrophages (CD163, IL-

1RA and CCL-18)[58]. Based on the evidence presented here, the accumulation of M2 macrophages in GD speculatively leads to a microenvironment permissive of carcinogenesis. Hepato-cellular carcinoma, NHL and multiple myeloma have been reported to be more common in GD[65;78;130]. These cancers affect the liver and bone marrow, major organs infiltrated by foamy macrophages in GD.

1.3.4.2 GAUCHER AND PSEUDO-GAUCHER CELLS

Macrophages, with identical appearances to Gaucher cells by light microscopy (pseudo-Gaucher cells), have been demonstrated in the bone marrow trephines of non-GD patients with MGUS[275] and multiple myeloma[246]. Lipid laden macrophages, therefore, may have a crucial role in supporting plasma cell expansion. Gaucher cells and pseudo-Gaucher cells have been suggested to acquire their morphological appearance from phagocytosed cells and the subsequent saturation of residual glucocerebrosidase activity[276;277]. The accumulation of pseudo-Gaucher cells, in GD-individuals, has been reported in the staging bone marrow trephines of individuals with other haematological cancers including Hodgkin lymphoma[278], NHL[279], chronic myeloid leukaemia (CML)[280], myelodysplasia[281] and common B-ALL[282]. The accumulation of pseudo-Gaucher cells, in one report, pre-dated the diagnosis of NHL, adding further speculation to their role in carcinogenesis[279].

The presence of pseudo-Gaucher cells, have been reported in other non-malignant haematological conditions that are associated with haemolysis and decreased red blood cell survival including thalassaemia[283] and sickle cell disease[284]. Glucosylceramide is a major component of cell membranes and macrophage glucocerebrosidase activity may become saturated in disorders associated with high cellular turnover. An association between myeloma and sickle cell disease has not been established, perhaps due to decreased life expectancy. There is one case series describing 6 Greek patients with sickle cell related haemoglobinopathies (ages 56-65) and myeloma in the literature[285]. A high incidence of paraproteinaemia was noted in a family of patients with congenital dyserythropoietic anaemia (3/20 MGUS; 1/20 Myeloma) but not in their relatives (n=10)[286]. However, co-inheritance of a cancer-gene, in individuals reported in this study, cannot be excluded.

Florena *et al.* (1996), using immuno-cytochemistry, demonstrated equivalent staining intensity for CD68 and MHC class II expression on Gaucher and pseudo-Gaucher cells. In addition, they found, that HLA-DR expression was expressed on all Gaucher cells, but on only 50% of pseudo-Gaucher cells[276]. Ultrastructural studies, using electron microscopy (EM), have demonstrated differences in structure between Gaucher cells and pseudo-Gaucher cells[278]. Furthermore, Gaucher cells have been suggested to express a different cytokine profile polarised towards a M2 phenotype[58]. Currently, it is unclear whether Gaucher cells, contribute to the elevated risk of multiple myeloma in GD. The liver contains the largest bulk of tissue macrophages in the human body. Therefore, as individuals with GD have been suggested to have an increased risk of hepatocellular carcinoma[287], the accumulation of Gaucher cells may be pathologically important. Those with homozygous N370S disease, associated with predominantly skeletal disease rather than organomegaly, have been suggested to be a subgroup of GD patients with a high risk of myeloma, perhaps due to an increased bone marrow burden of Gaucher cells[52]. Biological models are required to explore the contribution of Gaucher cells and pseudo-Gaucher cells in the development of carcinogenesis and plasma cell expansion.

1.3.4.3 OSTEOCLASTOGENESIS

Three cell types, osteoclasts, osteoblasts and osteocytes, contribute to bone remodelling[288]. Osteoblasts and osteocytes, derived from bone marrow mesenchymal cells, are involved in bone formation whereas osteoclasts digest mineralised bone via a ruffled cytoplasmic border[288]. Osteoclasts are highly specialised cells that originate either from the monocyte-macrophage lineage or are formed directly from granulocyte-macrophage colony forming units (CFU-GM)[289;290]. Morphologically, osteoclasts are large multi-nucleate cells found in Howship's lacunae with a diameter of 30-100μM, have abundant cytoplasm, contain azurophilic granules and stain positive for TRAP[197]. They express specific surface markers and are characterised by the presence of an F-actin ring plus the vitronectin and calcitonin receptors[289;291;292].

M-CSF stimulates the expansion and survival of osteoclast progenitors[289]. Osteoblasts, stromal cells and tumour cells secrete cytokines including M-CSF that facilitate the recruitment, commitment, differentiation and activation of osteoclast precursors[289]. The final common effector system mediating osteoclast differentiation

is the OPG (osteoprotegerin)-RANK (receptor activator of nuclear factor κB)-RANK-L (RANK-ligand) system. RANK-L, a member of the TNF-superfamily, binds to its receptor, RANK, on the surface of osteoclast progenitors. This leads to the up-regulation of several intracellular messengers, including Erk (extracellular signal-regulated kinases), p38, matrix metalloproteinase, PKC (protein kinase C), Akt, cfos and C-Jun, that mediate osteoclast differentiation and maturation[288;289]. RANK-L expression is inducible by parathyroid hormone and vitamin-D. RANK-L is expressed on the cell surface of stromal cells including osteoblasts and is secreted as a soluble factor by activated lymphocytes[274]. Genetically modified mice deficient in M-CSF or RANK-L develop osteopetrosis due to impaired osteoclastogenesis[293;294]. OPG, a dummy receptor secreted by stromal cells and osteoblasts, chelates free RANK-L leading to a microenvironment favourable to bone formation[288;295]. Knockout mice for OPG develop osteoporosis in adulthood[296].

Patients with multiple myeloma, Paget's disease and psoriatic arthropathy, conditions associated with abnormal bone resorption, are associated with elevated numbers of osteoclast progenitor cells within the peripheral blood[290]. Bone marrow osteoclasts, based on experimental and clinical data, have been found to promote plasma cell growth and survival[252;258]. Osteoclast numbers are increased in non-GD patients with monoclonal gammopathy with significantly more being present in the bone marrow of those with malignant disease[262]. Serum TRAP levels and hydroxyproline excretion (markers of bone turnover) have been shown to be elevated in individuals with multiple myeloma[297], confirming elevated osteoclast activity. Experimental models using conditioned medium from cultured plasma cells lines on rabbit osteoclasts have led to increased osteoclast activity and decreased osteoclast apoptosis[298]. This was found to be dependent on the production of M-CSF by immortalised plasma cells[298]. Patients with multiple myeloma have raised serum or tissue levels (in *vitro* or in *vivo*) of several cytokines capable of promoting osteoclastogenesis including RANK-L, IL-3, IL-6, stromal-derived factor 1α, TNF-α, MIP-1α[258;274;299]. The RANK-L/OPG ratio has shown to be comparatively higher in myeloma patients with severe bone disease or advanced stage[251]. Myeloma cells have been shown to induce RANK-L expression and to reduce OPG production in stromal cells plus osteoblasts[300]. Myeloma cells lead to a RANK-L/OPG ratio favourable to bony resorption by shredding CD138 (syndecan-1), which binds extra-cellular OPG[301].

Histological analysis has shown that osteolytic lesions occur adjacent to plasma cell infiltrates in the bone marrow specimens of patients with myeloma[274]. Abe *et al.* (2004), in a landmark paper, demonstrated the beneficial reciprocal interactions between osteoclasts and plasma cells[252]. They used a co-culture system consisting of osteoclasts derived from rabbit bone or generated post differentiation of human peripheral blood mononuclear cells (PBMCs) with RANK-L and M-CSF. Osteoclasts were shown *in vitro* to support myeloma cell survival (primary and cell line), rescue plasma cells grown in serum-depleted culture medium and to induce chemo-resistance in immortalised plasma cells to doxorubicin[252]. In addition, the authors demonstrated that the co-culture of plasma cells was sufficient for osteoclastogenesis, despite the absence of osteoclast differentiating cytokines[252]. Several other investigators have confirmed these findings with plasma cell expansion and osteoclastogenesis being enhanced in co-culture models[259;302]. Osteoclasts support myeloma cell survival and expansion by up-regulating the activity of intracellular signalling pathways (p44/42, STAT3 [Signal transducer and activator of transcription 3], MAPK [Mitogen-activated protein kinases]) and by the secretion of soluble factors including IL-6, osteopontin, BAFF (B-cell activating factor) and APRIL (a proliferation-inducing ligand)[252;258]. Yaccoby *et al.* (2005) showed that plasma cells become left shifted with plasmablastic morphology and a more immature immunophenotype post osteoclast co-culture. Furthermore, the authors demonstrated the development of dexamethasone chemoresistance in these de-differentiated cells[303].

Myeloma cells adhere to stromal cells or osteoclasts by the interaction of VLA (Very late antigen)-4 with VCAM (vascular cell adhesion molecule)-1. These interactions has been shown to lead to the production of MIP-1α and MIP-1β, potent osteoclast stimulating cytokines, from myeloma cells[304]. MIP-1α levels correlate with disease stage in patients (stage III>stages I, II)[305] and high levels of this chemokine have been associated with a poor prognosis[306]. Osteoclasts have been shown to support angiogenesis, which is enhanced further by the co-culture of human multiple myeloma cell lines[261].

Zoledronic acid, a potent inhibitor of osteoclastogenesis, has been shown to prolong survival in patients with multiple myeloma (Morgan *et al*, European Haematology Association abstract book, Barcelona, 2010), compared to a less potent oral bisphosphonate (clodronate). The addition of bisphosphonates to co-culture models (osteoclasts plus myeloma cells) has been shown to retard plasma cell expansion[252]. Therapies employed successfully in the treatment of multiple myeloma, including immuno-modulatory agents and proteasome inhibitors, have been shown to inhibit osteoclastogenesis in *in vitro* models[307-309].

The prolonged culture of circulating B-lymphocytes from myeloma patients in the presence of RANK-L has been shown to lead to osteoclastic differentiation. However, this was not seen in the peripheral cultures derived from controls. In addition, the prolonged culture of immortalised myeloma cell lines with RANK-L, has been reported by the same group to lead to osteoclastogenesis[310]. Furthermore, based on cytogenetic examination, myeloma cells have been suggested to fuse with osteoclasts to form hybrid cells[311]. Roato *et al.* (2005) demonstrated spontaneous osteoclast differentiation in cultured peripheral blood mononuclear cells from cancer patients with bony lesions, including those with multiple myeloma. In comparison to normal controls, the presence of osteoclast differentiating cytokines was not required[290].

Based on the data presented here, the OPG-RANK-RANK-L axis is a candidate pathway for the development of anti-myeloma drugs. Denosumab, a monoclonal antibody to RANK-L, failed to reduce the paraprotein level in relapsed or plateau phase patients with myeloma in a phase II trial[312]. However, it is currently unclear whether it will prove beneficial in combination with traditional chemotherapeutic regimes or solely as an agent for the treatment of myeloma bone disease. The therapeutic use of OPG analogues is also being explored[313].

Patients with GD have increased osteoclast numbers[61], in keeping with the high incidence of lytic lesions and osteoporosis[28]. TRAP, an acid phosphatase specific to osteoclasts, has been shown to be elevated in the serum of patients with GD[86]. However, many studies in GD, measuring markers of osteoclastic activity including deoxypyrindinoline and collagen C-terminal telopeptide, have failed to confirm

increased bone resorption[314;315]. As described above, elevated serum levels of osteoclast promoting factors, including M-CSF, IL-6, IL-8, HGF, MIP-1α and MIP-1β, have been reported in the literature of patients with GD[81]. Serum OPG levels have not been found to be decreased in patients with GD[316]. Prior results from our laboratory have suggested that monocytic precursors *in vitro*, derived from GD patients, are more sensitive to osteoclast differentiation and are capable of resorbing large areas of bone (personal communication, Dr Derralynn Hughes and Matthew Reed). GD patients have a large reservoir of accumulated macrophages within the bone marrow microenvironment[61]. Speculatively, these may lead to a pool of progenitor cells that are primed for osteoclastic differentiation. Literature is sparse regarding osteoclast pathology in GD. Osteoclastogenesis therefore seems a common feature to both multiple myeloma and GD. Further studies are required to assess whether GD patients generate a higher number of osteoclasts and whether these are supportive of the development and propagation of plasma cell clones. In addition, osteoclast function maybe disturbed in GD, given the macrophage origin of these cells. It is unclear at present whether GD patients with severe skeletal disease and radiographical evidence of osteoclast over-activity are at a higher risk of gammopathy.

1.3.4.4 OSTEOBLASTS

Evidence detailing abnormalities of osteoblastogenesis in the bone marrow microenvironment of myeloma patients has multiplied in recent years[317]. Yaccoby *et al.* (2006), utilising a co-culture system, showed that the proliferative and survival benefits conferred by osteoclasts on co-cultured primary plasma cells were attenuated by the addition of osteoblasts[318]. In addition, the authors demonstrated a reduction in myeloma tumour growth in SCID-hu mice injected with mesenchymal stem cells (MSCs). Engrafted MSCs led to osteoblastic differentiation and increased bone marrow density[318]. Co-culture studies have shown that osteoblast expansion, differentiation and function are inhibited by either the co-culture of myeloma cells or addition of their conditioned medium[260;300;319]. Dickkopf-1 (DKK-1), a soluble factor secreted by myeloma cells, inhibits osteoblast maturation, suppresses OPG production and increases RANK-L expression in osteoblasts[260]. DKK-1 inhibits the canonical Wnt pathway crucial to osteoblastogensis[300]. Serum DKK-1 levels are

higher in individuals with lytic bone disease, advanced myeloma and in those with malignant rather then benign gammopathy[320]. Li *et al.* (2008) elegantly showed that osteoblasts secrete decorin, a small leucine-rich proteoglycan that reduces myeloma cell survival, attenuates the stimulatory effects of osteoclasts on myeloma cell growth and retards the ability of plasma cells to induce osteoclastogenesis and the formation of vascular elements[319]. Secreted frizzled-related proteins, Sfrp-2 and sfrp-3, inhibitors of the Wnt pathway like DKK-1, impair osteoblast function and are manufactured by primary or immortalised plasma cells[300]. Furthermore the co-culture of myeloma cells leads to the down-regulation of the transcription factor RUNX2/CBFA-1 and OPG secretion in osteoblasts[321;322]. Giuliani *et al.* (2005) showed that the number of RUNX2/CBFA-1 cells in the bone marrow trephines of myeloma patients with lytic disease was greater than those without skeletal pathology[321].

As for osteoclasts, there is sparse literature regarding osteoblast pathology in GD. Speculatively, osteoporosis, may be secondary to impaired osteoid formation rather than enhanced osteoclastogenesis. Bone density has been shown to be decreased in the spine and hips of type I GD patients[323]. Serum biomarkers of osteoblastogenesis including type I procollagen and osteocalcin have been reported as elevated by some investigators[250;314] but normal by others[315;324]. It is unknown at present, if any, the degree of osteoblast pathology in GD or if present its contribution to the development of malignant gammopathy. A recent report, by Mistry *et al.* 2010, demonstrated severe osteoporosis, impaired osteoblastogenesis and decreased osteoblast differentiation in a GD mouse model[325]. They failed to show in their mouse model evidence of enhanced osteoclastogenesis.

1.3.4.5 CHRONIC INFLAMMATION

Chronic inflammation and immune stimulation are clearly important in the aetiology of carcinogenesis. Chronic bronchitis, inflammatory bowel disease, gastritis, hepatitis and chronic cholecystitis confer an elevated risk of lung, GI, liver and gall-bladder cancer respectively[266]. Osteoarthritis, ankylosing spondylitis and inflammatory arthropathies are associated with an elevated risk of paraproteinaemia[326]. Furthermore, Bacille Calmette-Guérin administration treats localised bladder malignancy by inducing an inflammatory response[266].

Inflammation is mediated by many different mediators, including prostaglandins, which are generated post degradation of arachidonic acid by cyclooxygenase (COX). Elevated COX-2 activity predicts for a worse prognosis in patients with GI-cancer. The use of non-steroid anti-inflammatory drugs (NSAIDs) and selective inhibitors of COX-2 are being explored in the treatment of bowel cancer[327]. Patients with rheumatoid arthritis, another chronic inflammatory disorder, have been reported to have a higher incidence of polyclonal gammopathy[328], MGUS and multiple myeloma[329]. In GD, deposited substrate is responsible for only 2% of visceral organ weight with large infiltrates of lipid-laden macrophages and inflammatory cells predominating[24]. As described above, pro-inflammatory cytokines including TNF-α, IL-6, IL-8 and IL-1 have been reported in the serum of patients with GD[73;74;76]. IL-6 is a major plasma cell proliferating cytokine[196]. Interestingly, a mouse model of GD demonstrated multi-system inflammation and evidence of B-cell expansion[330]. Speculatively, if inflammatory infiltrates are associated with gammopathy, then patients with high visceral load, or severe disease, may be at an elevated risk of developing plasma cell tumours.

Individuals with GD have a higher incidence of polyclonal gammopathy, MGUS and multiple myeloma suggesting that these conditions represent a continuum of disease. Shoenfeld *et al.* 1982 hypothesised that chronic immune stimulation was one plausible mechanism and that polyclonal stimulation pre-disposed activated plasma cells to clonal expansion[162]. However, follow-up in a cohort of non-GD patients failed to demonstrate that polyclonal gammopathy was a risk factor for developing a blood paraprotein[328].

1.3.4.6 CHRONIC ANTIGEN PRESENTATION

GD is a metabolic disorder that leads to disturbed sphingolipid metabolism resulting in the non-physiological accumulation and depletion of intermediate breakdown products. Sphingolipids are involved in cell signalling, cell membrane integrity, influence trans-membrane receptor activity and regulate apoptosis [19]. Ceramides, downstream products of glucosylceramide metabolism, facilitate apoptosis in cells exposed to chemotherapy or radiotherapy[19]. Glucosylsphingosine accumulates in enzymatic disorders of sphingolipid metabolism including GD[24]. One hypothesis pertaining to the development of gammopathy in GD is that of chronic antigenic

stimulation[66]. Here imbalances in sphingolipid metabolism lead to their non-physiological presentation on antigen presenting cells (APCs), facilitating an immune response favouring plasma cell expansion. Furthermore organ compromise may lead to the enhanced presentation of non-sphingolipid inflammatory antigens associated with tissue damage, leading to a microenvironment more favourable to B-cell proliferation. CD1d and MHC II class molecules, antigen presenting molecules, have been shown to have increased expression on the cell surface of GD monocytes[4]. However myeloid and plasmacytic derived dendritic cells (APCs) have been demonstrated to be decreased in the peripheral blood of patients with GD[331].

Glycolipids are the naturally occurring ligands for CD1d, a highly conserved molecule present on antigen presenting cells, including those derived from the monocytic lineage[332]. Processed sphingolipid, presented by CD1d tetramers on APCs, can expand and polarise invariant NK-T (iNKT) cells into either a Th1 or Th2 immune response[21]. CD1d complexed sphingolipid is presented to iNKT cells via their T-cell receptor that consists of an invariant alpha chain (Vα24Jα18) with preferential β chain pairings[21].

Alpha-galactosylceramide (αGalCer), derived from the marine sponge Agelas Mauritianus, is the first known ligand to expand iNK-T cells and has also been reported to be the most potent[332]. Endogenous glycolipids include isoglobotrihexosylceramide (iGB3)[333]. Knockout mice devoid of type I NK-cells (iNK-T cells) develop fibrosarcomas more readily when exposed to methylcholanthrene and the authors attributed this finding to impaired tumour survelliance[334]. Early phase trials in human patients with cancer have demonstrated only a transient benefit of αGalCer injections. However, the infusion of pre-loaded DCs with αGalCer has been suggested to result in an enhanced ability to expand iNK-T cells *in vitro*[333]. Many groups are exploring the potential of DCs, primed *in vitro* by tumour specific antigens, to regress human cancer via the stimulation of type I NK cells.

It is currently unclear whether glucosylceramide is a natural ligand for CD1d molecules. In addition, Ilan *et al*. (2009) argued, based on experimental data, that

glucosylceramide administration polarises iNKT cells towards a Th2 response. They cited the beneficial effects of glucosylceramide administration in animal models of auto-immunity, malignancy and inflammatory disorders[20]. Failings in their argument include that the chronic antigenic stimulation of plasma cells in GD maybe mediated by the accumulation of secondary substrates. Furthermore it is currently unknown whether pathological quantities of glucosylceramide confer the same biological effect as those seen at lower levels. Other investigators have speculated that glucosylceramide may out-compete natural ligands such as iGB3 or that CD1d molecules are ineffectively loaded with lysosomal sphingolipids[335]. The accumulation of secondary substrates, including glucosylsphingosine, may be more important mechanistically in the development of gammopathy in GD.

Speculatively, pathological quantities of glucosylceramide, could promote Th2 polarisation of type I NK cells, resulting in a cytokine profile favourable to the development of a plasma cell clone.

1.3.4.7 IMPAIRED TUMOUR SURVELLIANCE

The discriminating features of cancer cells are that they undergo autonomous proliferation, ignore inhibitory growth signals, invade adjacent tissues, promote angiogenesis and escape cell death due to dysregulation of apoptotic mechanisms[336]. In addition, the concept of impaired tumour-surveillance as a facilitator of tumour growth and chemo-resistance has gained increasing support in recent years[337-339]. This is highlighted by the fact that patients with human immunodeficiency virus (HIV) have an elevated risk of malignancy, including haematological cancer and multiple myeloma[340]. Furthermore, the level of paraproteinaemia has been suggested to decline with anti-retroviral therapy in HIV[341]. Reduced B-cells, CD4+ve T-cells and cytotoxic T-cells have been reported to confer a poor prognosis in multiple myeloma, perhaps due to the immune escape of tumour cells[339]. A retrospective study of US veterans case-notes, based on ICD (international classification of disease) codes, demonstrated that a prior history of autoimmune disease (including pernicious anaemia and haemolytic anaemia) was a risk factor for developing MGUS or multiple myeloma[326]. Chediak-Higashi syndrome is caused by mutations within the lysosomal trafficking regulator gene (CHS1/LYST). Patients with this syndrome have dysfunctional cytotoxic T-cells, abnormal NK cells, recurrent infections and an

increased incidence of malignancy[336]. Imai *et al.* (2000) demonstrated in a prospective trial of 3,625 individuals that low peripheral lymphocyte cytotoxic activity was associated with a higher risk of developing cancer[342]. In addition, it has been shown that individuals with breast cancer have lower peripheral NK activity on calcein-AM release assays. Furthermore the same authors showed that individuals with HER2+ve (Human Epidermal Growth Factor Receptor 2) breast cancer (confers a poor prognosis) had lower NK activity than that seen in HER2-ve individuals[343]. Patients with myeloma have been shown to have impaired NK cell activity[344].

It is unknown at present whether lysosomal dysfunction pre-disposes to an elevated risk of malignancy. The literature is devoid of case series describing an elevated risk of cancer in LSDs other than GD.

Early evidence supporting the idea that GD lymphocytes may be functionally abnormal is derived from transmission electron microscopy (TEM) studies describing storage deposition[345]. EBV-transformed lymphoid cells from patients with GD have been shown to have reduced glucocerebrosidase activity (15-30% of normal) and to accumulate sphingolipid[346]. Marti *et al.* (1988) found the absolute lymphocyte count (ALC) to be marginally elevated in 6/7 untreated patients with type I GD together with an increase in the number of peripheral CD20+ve (B-cells), CD4+ve and CD8+ve lymphoid cells[139]. The authors also described a stastically non-significant decrease in the percentage of CD57+ve and CD16+ve NK cells. Although, the percentage of NK cells was reported to be reduced, a higher absolute number of NK cells was noted[139]. There have been several other reports, many contradictory, detailing CD4, CD8 and NK-T cell subsets in patients with GD [4;347;348]. In contrast to Marti *et al*. (1988), some investigators have shown a decrease in the absolute number of CD4+ve and CD8+ve T-cells, whereas others have reported normal percentages in treatment-naïve patients[4;347]. Early reports of T-cell dysfunction include impaired E rosetting[349] and abnormal responses to lectin stimulation[348]. However, for the past two decades the literature is devoid of reports detailing functional T-cell anomalies.

Macrophages play a critical role in tumour surveillance, including the stimulation of cytotoxic γδ T-cell and NK-cells. In addition, they mediate antibody-dependent cytotoxicity and present tumour antigens on MHC and CD1d molecules. Lipid-laden Gaucher cells have been demonstrated to have a Th2 or M2 phenotype which has been reported in histological specimens of non-GD patients to be associated with tumour progession[268].

Dendritic cells (DCs), potent antigen presenting cells, present cancer-derived antigens on MHC-I, MHC-II and CD1d molecules. It has been demonstrated that patients with GD have a reduction in the number of myeloid and plasmacytoid-derived peripheral blood DCs. In addition, the authors showed that patients with GD were unable to expand monocyte-derived DCs in culture[331]. The frequency of peripheral blood DCs is also decreased in patients with Fabry disease, suggesting lysosomal dysfunction as a common aetiology[350]. Abnormalities of DCs may confer impaired tumour surveillance.

Currently, our understanding of lymphocyte anomalies in GD is insufficient to ascertain whether they could contribute to a microenvironment more conducive to cancerous expansion. Patients with GD have an elevated risk of malignancy[287] and further studies are required to delineate whether glucocerebrosidase-deficient cells undergo oncogenic transformation more readily or whether their expansion is favoured by impaired elimination, secondary to defects in innate and adaptive immunity.

1.3.5 *GBA1* MUTATION STATUS – GAMMOPATHY

It is currently unclear why patients with GD have a higher incidence of myeloma[130]. Interestingly, in recent years, individuals who are heterozygotes for a *GBA1* mutation have been shown to have a higher incidence of Parkinson's disease[351]. Misfolded mutant *GBA1* alleles are associated with the induction of endoplasmic reticulum stress plus cellular dysfunction[16] and this has been suggested to result in toxicity to substantia nigra cells[352]. Speculatively, heterozygotes for Gaucher gene mutations, may have an elevated incidence of plasma cell dyscrasias. However, if an association were proven then co-inheritance of a closely located cancer gene to *GBA1* on

chromosome 1 would require exclusion. Speculatively, hypotheses relating to chronic inflammation, substrate deposition, macrophage accumulation, immune abnormalities and a permissive bone marrow microenvironment would become less credible if carrier status was linked to gammopathy. However, methodology depending, *GBA1* heterozygotes, have been reported to have residual glucocerebrosidase activity that overlaps with both homozygotes and normal controls[353]. Carriers may have "intermittent lipid status" and it is unknown at present whether cellular dysfunction or disturbed sphingolipid metabolism in these individuals is sufficient to confer gammopathy.

In a study of 95 patients with myeloma (18/95 Ashkenazi Jews), 2 Jewish patients were found to be carriers of a N370S allele[354]. However, this study was of insufficient power to assess for an association between *GBA1* mutation status and a malignant plasma cell disorder. As discussed above, N370S homozygotes have been reported to have a higher incidence of multiple myeloma compared to patients who are heterozygous for a N370S allele[52]. These findings, if confirmed in other cohorts, raise questions on a cellular level regarding the pathophysiological disturbances imparted on cells by different mutations.

1.3.6 PROJECT AIMS AND HYPOTHESIS

Despite several clinical papers detailing immunoglobulin abnormalities in GD patients, few have investigated potential aetiologies. In addition, over the past 2 decades, novel disturbances within the bone marrow microenvironment and immune system have been described in non-GD patients with multiple myeloma. These abnormalities have yet to be explored in GD and may be similar. The aims of this thesis are:-

(1) To identify whether carriers for a *GBA1* mutation, as GD patients, have a higher than expected incidence of gammopathy.
(2) To identify severity markers in patients with GD that predict for gammopathy.
(3) To identify abnormalities within the GD microenvironment that pre-dispose to gammopathy.

These aims are explored in the chapter hypotheses stated below:-

Chapter 3

Hypothesis 1:- Disease severity predicts for monoclonal and polyclonal gammopathy in GD

Rationale:- Several cohorts of GD patients have been shown to have an increased incidence of gammopathy. Prior literature has been inconclusive and not sufficient to answer whether markers of disease severity including biomarkers predict for immunoglobulin abnormalities.

Methods:- Analysis of clinical data pertaining to local patients with GD at the Royal Free Hospital.

Hypothesis 2:- *GBA1* is a cancer gene or B-cell promoter and carrier status leads to an elevated risk of paraproteinaemia.

Rationale:- Patients with *GBA1* mutations, homozygotic or heterozygotic, have an elevated risk of Parkinson's disease. It is currently unknown whether carrier status confers an increased risk of monoclonal gammopathy.

Methods:- *GBA1* gene analysis of non-GD patients who have an established paraprotein from Jewish descent.

Hypothesis 3:- Patients with myeloma acquire pathology in the monocytic lineage similar to that seen in GD.

Rationale:- Non-GD individuals with haematological malignancies including multiple myeloma have infiltrates of foamy macrophages by light microscopy on bone marrow examination (pseudo-Gaucher cells). It is unclear whether these cells or their precursors acquire similar biochemical disturbances to monocytic cells derived from patients with GD.

Methods:- Monocyte glucocerebrosidase activity and plasma chitotriosidase activity.

Chapter 4

Hypothesis 1:- GD compared to non-GD derived monocytic cells preferentially support plasma cell expansion and survival.

Rationale:- Co-culture experiments in non-GD patients have demonstrated that osteoclasts and macrophages promote plasma cell survival. Osteoclast cultures have been shown to expand plasma cells.

Methods:- Plasma cell co-culture experiments utilising adherent monolayers derived from controls and GD patients.

Hypothesis 2:- GD compared to non-GD derived macrophages/osteoclasts protects myeloma cells from chemotherapy-induced apoptosis.

Rationale:- Co-culture experiments of non-GD patients have shown that macrophages and osteoclasts reduce the sensitivity of plasma cells to chemotherapeutic drugs. Furthermore there are anecdotal reports of poor responses to chemotherapy in GD patients with myeloma.

Methods:- Plasma cell co-culture experiments utilising adherent monolayers derived from controls and GD patients.

Hypothesis 3:- GD macrophages precursors are primed for osteoclastic differentiation which is potentiated by plasma cell co-culture

Rationale:- Plasma cell co-culture promotes osteoclast formation in macrophage cultures derived from non-GD patients. Osteoclasts and plasma cells co-localise in the bone marrow specimens of non-GD patients and have been suggested to reciprocally facilitate the growth and survival of each other.

Methods:- Plasma cell co-culture experiments utilising adherent monolayers derived from controls and GD patients.

Hypothesis 4:- Compared to control cultures GD derived macrophages/osteoclasts are more efficient at rescuing myeloma cells pre-treated with chemotherapy.

Rationale:- Co-culture experiments in non-GD patients have shown that macrophages and osteoclasts rescue plasma cells pre-treated with chemotherapy.

Methods:- Plasma cell co-culture experiments utilising adherent monolayers derived from controls and GD patients.

Chapter 5

Hypothesis 1:- GD lymphocytes have low glucocerebrosidase activity, accumulate substrate and have abnormalities within their lymphoid repertoire that contribute to a microenvironment favourable to carcinogenesis.

Rationale:- Non-GD patients with abnormalities in their lymphocyte count or subsets including NK cells have an increased incidence of malignancy.

Methods:- Local patient data, immunophenotyping and electron microscopy.

Hypothesis 2:- GD patients due to disturbances in sphingolipid metabolism and glycolipid presentation have abnormalities in iNK-T cell expansion. This contributes to a microenvironment conducive to malignancy.

Rationale:- Numerical low numbers of invariant NK-T cells are seen in individuals with malignancy.

Methods:- Sphingolipid stimulation assays, cell culture.

Hypothesis 3:- GD patients due to impaired NK-function have impaired tumour surveillance.

Rationale:- Decreased peripheral NK-cell lytic activity is associated with impaired tumour surveillance in non-GD individuals.

Methods:- NK killing assays.

Hypothesis 4:- Patients with GD have numerical and function abnormalities within their peripheral blood cells that lead to impaired tumour surveillance.

Rationale:- Non-GD patients with impaired peripheral blood killing assays have an elevated risk of malignancy.

Methods:- Peripheral blood killing assays.

2 GENERAL METHODS

2.1 PATIENT AND HEALTHY CONTROL RECRUITMENT

This study was approved by the Royal Free Hospital Multi-Centre Research Ethics Committee (MREC) to recruit healthy controls or individuals with either Gaucher disease or a blood paraprotein (myeloma, MGUS or non-specified). In addition, site-specific approval was obtained from the Local Research Ethics Committee (LREC) and Research and Development (R&D) departments in the following NHS hospital trusts:-

- Royal Free Hampstead NHS Trust
- Barnet and Chase Farm Hospitals NHS Trust
- North West London Hospitals NHS Trust
- Barking, Havering and Redbridge University Hospitals NHS Trust

All patients received an information sheet 14 days prior to providing signed informed consent (See Appendix A; example information sheet/consent form). Recruited individuals were assigned a unique identification code. Individual patient data were stored within a secure location, on a password protected system and access was limited to study investigators. Tissue specimens (DNA and bone marrow) were acquired and stored in accordance with the Human Tissue Act.

2.2 GENERAL CELL CULTURE METHODS

2.2.1 CELL CULTURE

Primary cells and cell lines (K562, NCI-H929, U266 and JJN3) were maintained in R10 growth medium, which contained RPMI 1640 (Sigma-Aldrich, Poole, UK), 10% heat-inactivated foetal calf serum (Invitrogen™, Paisley, UK), 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (Invitrogen, Paisley, UK), 2mM L-glutamine (Sigma Aldrich) and penicillin/streptomycin (>10,000U/ml penicillin, >10mg/ml streptomycin; Sigma Aldrich). Cells were incubated in a humidified atmosphere at 37°C and 5% CO_2 (Haraeus Instruments, Michigan, US). Growth medium was changed in all cell lines the day before use to ensure that cells were in the exponential phase of growth. Osteoclast medium refers to R10

supplemented with the osteoclast differentiating cytokines Receptor Activator for Nuclear Factor κ B Ligand (RANK-L; 30ng/ml; Peprotech EC, London, UK) and macrophage colony stimulating factor (M-CSF; 25ng/ml; Genscript Co-operation, NJ, USA).

2.2.2 IMMORTALISED CELL LINES

(a) U266 – Generously donated by Dr Popat, Institute of Cancer, Barts Cancer Centre, UK (St Bartholomew's Hospital). This is a non-adherent, IgE lambda human multiple myeloma cell line with a doubling time of approximately 55 hours. Cell density was maintained between $0.2x10^5$-$1.5x10^6$ cells/ml. Cells were grown in R10 with twice weekly cell passage. Immuno-phenotyping confirmed CD138 positivity but the absence of CD38, another plasma cell marker (Figure 2-1).

(b) NCI-H929 – Generously donated by Dr Ellie Necheva, Academic Haematology, Royal Free Hospital, UK. This is a non-adherent human multiple myeloma cell line and is IgA secreting. Doubling time is approximately 70 hours and subcultures were performed twice weekly in order to maintain a cell density of approximately $1x10^6$ cells/ml. In this NCI cell line CD38 was expressed by only a minority of cells (Figure 2-1).

(C) JJN3 – Generously donated by Dr Ellie Necheva, Academic Haematology, Royal Free Hospital, UK. This is a sub-clone of the cell line JJN-1 that originated from a patient with IgA1κ plasma cell leukaemia. JJN-3 is a non-adherent cell line composed of mainly uni-nucleate oval shaped cells. Doubling time is 20-35 hours and cell densities were maintained between 0.2-$2x10^6$cells/ml. CD138 positivity confirmed the cell line to be of plasma cell origin.

(D) K562 - Generously donated by Janet North, Academic Haematology, Royal Free Hospital, UK. This cell line was derived from a patient with chronic myeloid leukaemia in blast crisis and harbours the *BCR-ABL* fusion gene (Philadelphia chromosome). K562 cells are non-adherent and were maintained with a density of between 0.1-$1.5x10^6$ in R10 medium. Doubling time is approximately 30 hours.

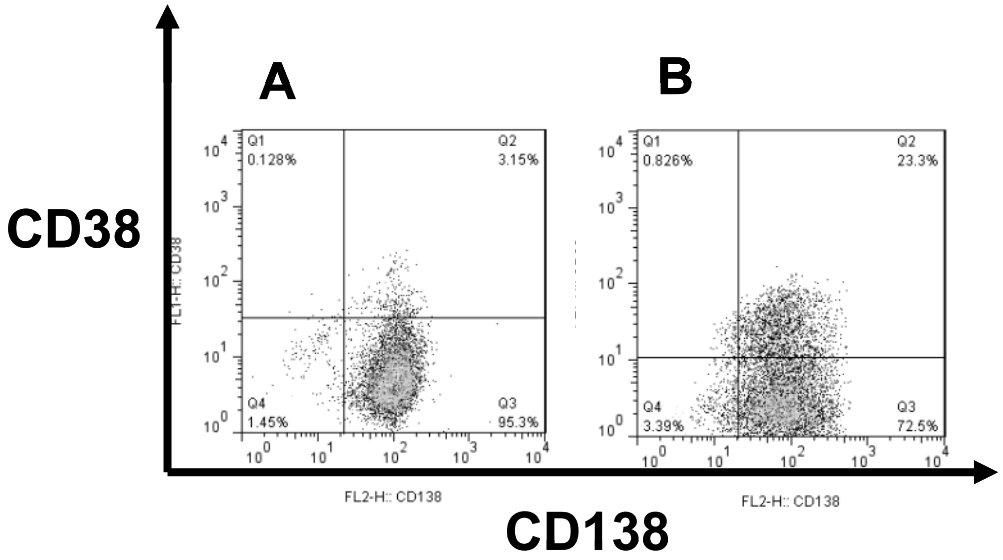


Figure 2-1. CD138/CD38 expression of the (A) U266 and (B) NCI-H929 cell lines.

2.2.3 CELL COUNTS AND VIABILITY

Cells were counted by light microscopy using a haemocytometer with an etched grid system. 10µl of cell suspension was added to 10µl of 0.2% trypan blue (diluted in filtered phosphate buffered saline, PBS; Sigma Aldrich) for 1 minute in a micro-centrifuge tube. Live cells are colourless and dead cells stain blue, as they are unable to exclude dye due to impaired membrane integrity. A minimum of 200 cells was analysed. The viability of all cell lines was maintained at greater than 90%.

2.3 THAWING AND FREEZING OF CELL LINES

2.3.1 LIQUID NITROGEN STORAGE OF CELL LINES

The prolonged culture of immortalised cell lines can lead to baseline changes in their growth characteristics, morphological appearance and chemosensitivity secondary to genotypic drift. Therefore, stocks of cryopreserved cells, frozen on the same date, were used for each cohort of experiments. Growth medium (R10) was changed the day before cryopreservation. Cells were spun (300*g*, 10 minutes) and re-suspended in freezing medium consisting of 90% Foetal Calf Serum (FCS) and 10% dimethyl sulfoxide (DMSO; Sigma Aldrich) at a concentration of $5x10^6$cells/ml. Vials were immediately transferred to a freezing container containing iso-2-propanolol (Sigma-Aldrich), to allow slow cooling (1°C per minute), and placed in a -80°C freezer. The following day, cryovials (Jencons, East Grinstead, UK [division of VWR]) were stored in liquid nitrogen until required.

2.3.2 THAWING CELLS FROM LIQUID NITROGEN

Cryovials containing $5x10^6$cells/ml were recovered from liquid nitrogen and thawed in a pre-warmed water bath. Cells were transferred carefully into 14mls of warmed growth medium (R10, 37°C) before gentle centrifugation at 250*g* for 5 minutes. Cells were re-suspended in 3mls growth medium and placed in a cell incubator for 4 days prior to splitting.

2.4 SAMPLE PREPERATION

2.4.1 SEPERATION OF PERIPHERAL BLOOD MONO-NUCLEAR CELLS (PBMCS)

Blood (20mls) was collected into a sterile universal container with 200µl of preservative free heparin (5,000U/ml, CP pharmaceuticals Ltd, Wrexham, UK) before being inverted several times to ensure adequate mixing. Heparinised blood was mixed with 10mls of 3% Dextran-500 (Fisher Scientific, Loughborough, UK) and incubated for 45 minutes in a pre-heated waterbath at 37ºC. PEG was made up with 1L PBS supplemented with 1mM ethylenediaminetetraacetic acid (EDTA; Sigma Aldrich) and 10mM glucose (Sigma Aldrich). Isolated fractions (supernatant and non-supernatant) were supplemented with PEG to a total volume of 20mls before being carefully pipetted onto a layer of 12mls Ficoll-Pague™-Plus (GE Healthcare, Chalfont St Giles, UK). Samples were centrifuged at 650*g* at 4ºC for 25 minutes (brake off). Following gradient centrifugation, the white cell layer was removed using a sterile pipette and recovered cells were washed twice in 50mls of PEG (centrifuged for 10 minutes at 4ºC, at 400*g*). Cells were re-suspended in 5mls of R10 medium.

2.4.2 PREPERATION OF CELL SUSPENSIONS FOR TRANSMISSION ELECTON MICROSCOPY

PBMCs were isolated as described above before being re-suspended in 1ml of refrigerated fixative solution (20mls of 20% paraformaldehyde [Sigma Aldrich], 16mls of 25% glutaldehyde [Sigma Aldrich] and 59mls of PBS). Cells were stored for a minimum of 2 hours at 4°C before being centrifuged in a microcentrifuge tube

to form a pellet. Cells were washed twice in PBS and incubated for 10 minutes in staining solution (1% osmium tetroxide [TAAB Laboratories Equipment, Aldermaston, UK], 1.5% potassium ferricyanide [TAAB Laboratories Equipment], PBS). Cells were washed 5 times in distilled water and dehydrated with increasing concentrations of ethanol (30%, 50%, 70%, 90% and 100%). Samples were left in a mixture of 50% alcohol/50% Lemix epoxy resin (TAAB Laboratories Equipment) on a mixer overnight.

The following day, resin infiltrated samples were placed in 100% Lemix resin for at least 4 hours before being embedded in 100% Lemix Resin overnight. Semi-thin (1µm) sections were cut using glass knives on a Reichert-Jung Ultracut microtome before being stained with 1% Toluidine Blue overnight (TAAB Laboratories Equipment). Ultra-thin sections were cut using a diamond knife (Diatome; TAAB Laboratories Equipment) and collected on 300HS 3.05mm copper grids (Gilder Grids, Grantham, UK). Sections were stained with saturated alcoholic uranyl acetate (TAAB Laboratories Equipment) for 5 minutes and transferred into Reynold's lead citrate (TAAB Laboratories Equipment) for a further 5 minutes. Specimens were viewed and photographed using a Phillips CM120 transmission electron microscope (Philips, Eindhoven, Netherlands). Specimens were prepared by Royal Free Hospital, Electron Microscopy Department.

2.4.3 PREPERATION OF PLASMA SAMPLES

Blood, collected into tubes containing EDTA, were centrifuged at 3500rpm for 5 minutes at 4°C. Aspirated supernatant was stored in microcentrifuge tubes (Fisher scientific) at -80°C until further use.

2.5 FLOW CYTOMETRY ANTIBODIES

2.5.1 GENERAL FLOW CYTOMETRY

Throughout this project, monoclonal antibodies were used as direct conjugates with fluorescein isothiocyanate (FITC), R-Phycoerythrin (PE) plus peridinin-chlorophyll-protein complex (PerCP) and following fluorochrome excitation, emitted light was detected in the FL1, FL2 and FL3 channels respectively (Table 2-1). Generally, 0.1-

$1x10^6$/100µl cells were incubated in the dark with an antibody cocktail for 15-30 minutes before washing twice in PBS. Cells were re-suspended in PBS (accept for AnnexinV, see below) or FACSflow fluid (Becton Dickinson, BD, Oxford, UK) prior to analysis.

TARGET	SPECIES	IG CLASS	PRODUCT CODE	COMPANY	CLONE	CONJUGATE	DILUTION/ INCUBATION TIME
Human CD3	Mouse	IgG1k	345766	BD	SK7	PerCP	1:20 15-30 minutes
Human CD4	Mouse	IgG1k	345768	BD	SK3	FITC	1:10 15 minutes
Human CD8	Mouse	IgG1k	345773	BD	SK1	PE	1:10 15 minutes
Human CD14/64	Mouse	IgG2b,k IgG1	333179	BD	MψP9 MD22	FITC/PE	1:10 15 minutes
Human CD56	Mouse	IgG2b,k	345812	BD	NCAM16.2	PE	1:5 15 minutes
Human CD56	Mouse	IgG1k	345810	BD	MY31	PE	1:5 15 minutes
Invariant NK-T Cell (6B11)	Mouse	IgG1k	558371	BD	6B11	FITC	1:5 30 minutes
Human CD38	Mouse	IgG1k	555456	BD	HIT2	FITC	1:10 10 minutes
Human CD138	Mouse	IgG1k	552026	BD	Mi15	PE	1:10 10 minutes
Human CD1d	Mouse	IgG1k	550254	BD	CD1d42	PE	1:5 30 minutes
Human CD19	Mouse	IgG1k	345777	BD	4G7	PE	1:5 30 minutes
BrdU	Mouse	IgG1k	347583	BD	B44	FITC	1:5 30 minutes
AnnexinV	-	-	130-093-060	Miltenyl Biotec	-	FITC	1:10 15 minutes

Table 2-1. Antibodies utilised in flow cytometry.
BD, Becton Dickinson.

Stained samples were kept on ice if there was a delay in analysis. Samples were acquired on a FACScan (BD) and analysed with CellQuest™ (BD) or FlowJo 7.6.1 software (©Tree Star, Inc., Oregon, USA). Typically 5,000-10,000 gated events were collected for analysis.

2.5.2 ANNEXIN V/PROPIDIUM IODIDE FLOW CYTOMETRY ASSAY

In healthy cells, phosphatidylserine (PS), a negatively charged membrane phospholipid, is located to the cytoplasmic side of the lipid bi-layer. PS is translocated to the extracellular surface of the cell membrane during early apoptosis and the additional uptake of the nucleic acid stain propidium iodide (PI) signifies late apoptotic cells that are unable to exclude PI due to breakdown in their cellular and nuclear membranes. AnnexinV has high affinity to PS in the presence of binding buffer rich in calcium ions. This assay therefore is able to identify both an early apoptotic population (annexinV+ve, PI-ve) and a necrotic/dead subpopulation (annexinV+ve, PI+ve).

Cell concentration varied between 0.25-1x10^6 per ml depending on the number recovered from individual experiments. Cells were washed twice in annexin binding buffer (10mM HEPES, 150mM NaCl [Sigma Aldrich], 5mM KCl [Sigma Aldrich], 1mM $MgCl_2$ [Sigma Aldrich] and 1.8mM $CaCl_2$ [Sigma Aldrich] buffered at pH 7.2) and centrifuged at 400*g* for 5 minutes before being re-suspended in 100µl of binding buffer. 10µl of FITC-labelled annexinV was added to samples before incubation in the dark at room temperature for 15 minutes. Samples were washed twice in annexinV binding buffer before being re-suspended in a final volume of 500µl. Immediately prior to analysis, 5µl of PI solution (100µg/ml ddH_20; Fluka, Sigma Aldrich) was added. PI stock solution, 1mg/ml in distilled water, remained stable for 6 months at 4°C. On excitation, PI, emits fluorescent light with a wavelength (617nm) detectable in FL2.

2.5.3 BRDU ASSAY – U266 CELLS – FLOW CYTOMETRIC ASSAY

Bromodeoxyuridine (BrdU) is a uridine analogue that is incorporated into DNA instead of thymidine during the S-phase of cell replication. Culture cells are pulsed with BrdU for a pre-designated time period prior to being fixed, permeabilised and stained with mouse anti-BrdU IgG1 FITC (BD Biosciences). Combined staining with the nucleic acid stain PI allows the percentage of cells in G0/G1, S and G2/M phases of the cell cycle to be quantified (Figure 2-2).

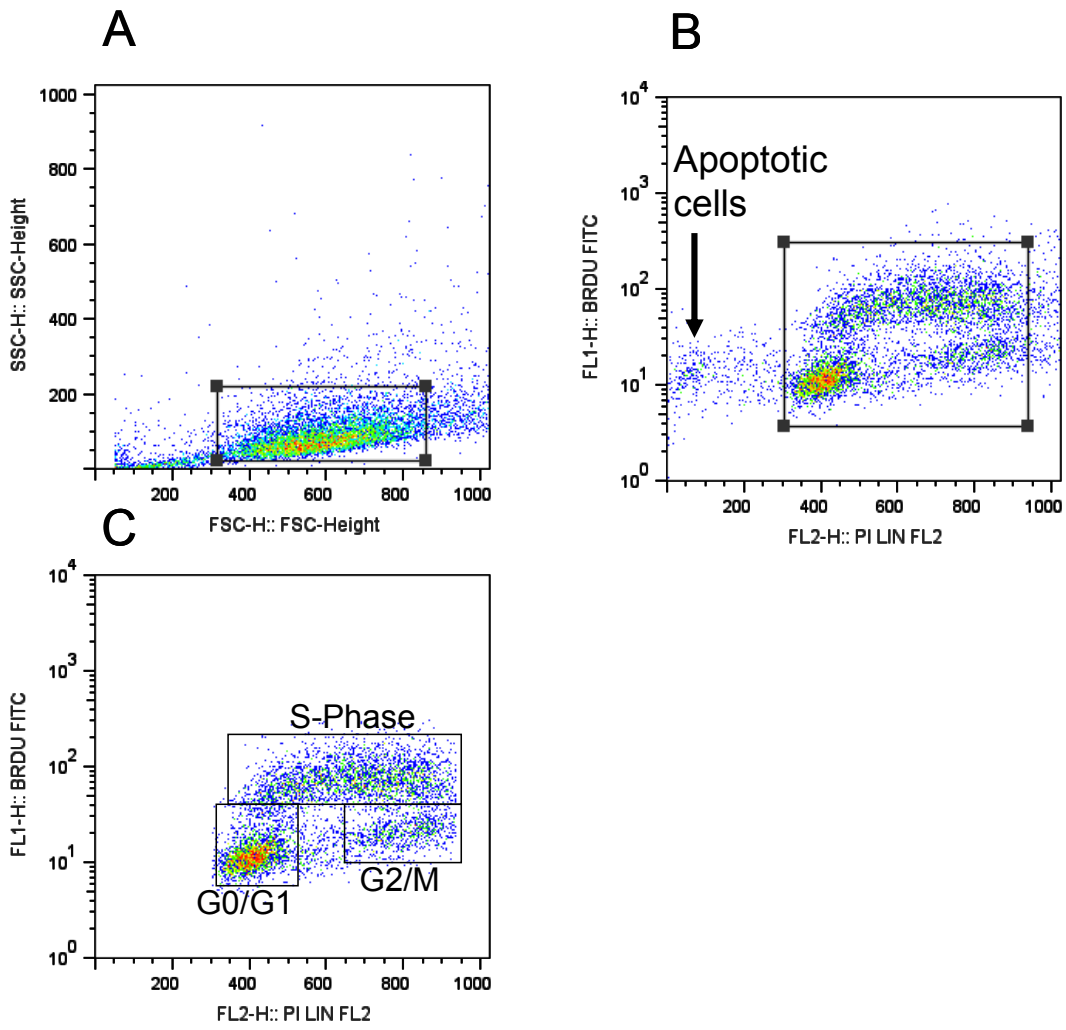


Figure 2-2. Representative cell cycle obtained from U266 cells pulsed with BrdU for 4 hours. Live cells were gated on (A, B) prior to quantifying the % of S, GO/G1 and G2/M phase cells.

BrdU (Sigma Aldrich) was added to experimental wells to achieve a final concentration of 20-30µM before being placed in the cell incubator for 1-4 hours (specific time detailed for each experiment). Recovered cells were washed twice (centrifuged at 500*g*, room temperature, for 15 minutes) in 1% BSA (bovine serum albumin, Sigma Aldrich)/PBS. Cells were then re-suspended in 200µl of ice cooled PBS and added slowly to a vortex of 70% ethanol (pre-chilled to -20ºC). Samples were placed on ice for 30 minutes and then centrifuged for 10 minutes at 400*g* (10ºC). The supernatant was carefully removed to leave a pellet of fixed cells. The pellet was loosened by vortexing before being re-suspended in 1ml of 2N HCL/Triton X-100 (Sigma Aldrich) and incubated at room temperature for 30 minutes.

The denatured fixed cells were centrifuged at room temperature (400*g* for 10 minutes) before being washed in 0.1M sodium borate solution, pH 8.5 (Sigma Aldrich). Cells were centrifuged again at 400*g* for 10 minutes at room temperature before suspension in 100µl of 0.5% Tween 20 (Sigma Aldrich)/1%BSA/PBS solution. 10µl of anti-BrdU FITC was added and cells were incubated in the dark for 30 minutes at room temperature.

After washing once in 0.5%Tween/1%BSA/PBS, cells were re-suspended in 1ml of PBS containing 5µg/ml of PI and left in the dark for a further 15 minutes. Cell cycle analysis was then performed by flow cytometry.

2.5.4 FLOW CYTOMETRY BETA-GLUCOCEREBROSIDASE ASSAY

Lysosomal β-glucocerebrosidase was determined as described previously (Rudensky *et al* 2003[355]; Havenga *et al*. 1998[356]). The biochemical basis of this assay is the cellular uptake and enzymatic degradation of the substrate fluorescein di-β-D-glucopyranoside (FDGlu; Invitrogen) by β-glucocerebrosidase and results in the production of a cellular fluorochrome that is detectable in the FL1 channel. This assay was made specific for lysosomal glucocerebrosidase by comparison to a paired sample pre-treated with conduritol-B-epoxide (CBE; Sigma Aldrich), an inhibitor of lysosomal β-glucosidase activity. Aliquots of FDGlu (20mM, DMSO) and CBE (50mM, dH_20) were stored at -20°C until required. Leucocytes, isolated from peripheral blood, were re-suspended at a concentration of 10^5-10^6 cells/ml in RPMI1640 with 2% FCS (R2). Two aliquots of 100µl cell suspension were placed in test tubes before one aliquot was incubated with 2µl of 50mM CBE (Sigma Aldrich) for 1 hour at room temperature. Following the addition of 100µl of R2, cells were incubated at 37°C with 10µl of 20mM FDGlu for 1 minute and diluted with an extra 780µl of R2 before incubation for 45 minutes at room temperature.

Glucocerebrosidase activity was determined by the median fluorescent intensity (MFI) of liberated fluorochrome from the carrier substrate FDGlu and was expressed as a ratio of the sample MFI to that obtained from a paired sample (from the same individual) treated with the lysosomal glucocerebrosidase inhibitor, CBE.

Monocytes and lymphocytes were identified based on their forward and side-scatter characteristics. Lymphocyte subsets were identified by CD3, CD56 and CD19 co-immunostaining in the FL2 and FL3 channels.

2.5.5 TO-PRO®-3 IODIDE NUCLEIC ACID STAINING

TO-PRO®-3 iodide (1mM, DMSO, stored -20°C; Invitrogen) was thawed and 500µl was added to 50mls of Hank's buffered salt solution (HBSS; Invitrogen, 10µM stock solution). Stock solution was added to cells in a 1:10v/v ratio before analysis in the FL4 channel (excitation wavelength 642nm; emission wavelength 661nm; He-Ne laser).

2.6 OSTEOCLAST CULTURE

2.6.1 OSTEOCLAST ASSAY

PBMCs were separated from whole blood (20-40mls) as described above and the number of cells isolated was determined. Between 0.5-1.0x10^6 PBMCs were stained with CD14/64 before undergoing FACs analysis to determine the monocyte percentage (Figure 2-3).

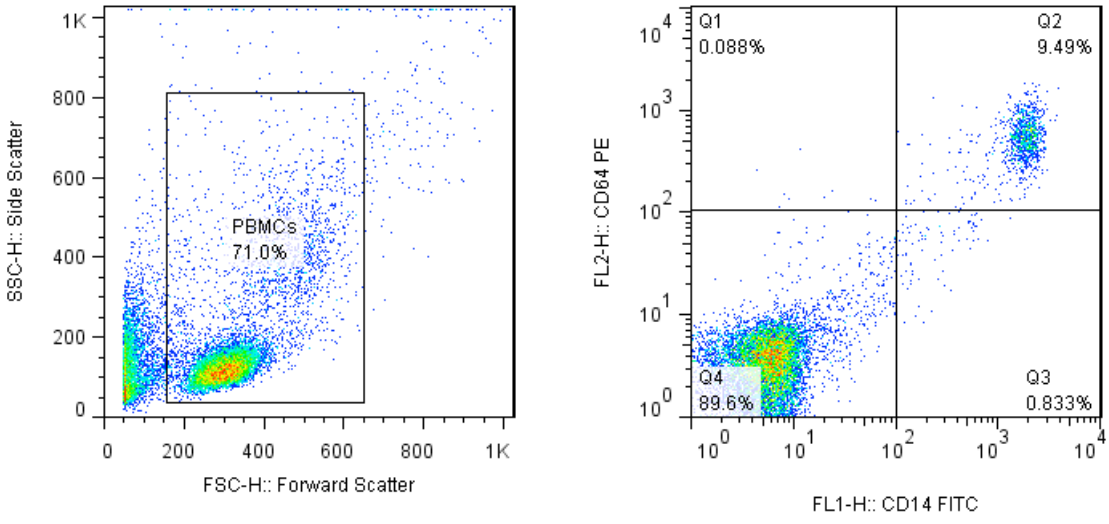


Figure 2-3. Flow cytometry for CD16/64. The percentage of monocytes is indicated by the CD14+ve/CD64+ve fraction in the upper right quadrant.

PBMCs were re-suspended in R10 medium to give a monocyte concentration of $0.5x10^6$/ml. Osteoclast cultures were grown in 24 well plates. In each experimental well, 3 ethanol-sterilised coverslips were inserted (6mm diameter, Richardsons of Leicester Ltd, UK). These were subsequent recovered, fixed and stained using a histo-chemical technique for osteoclasts (tartrate resistant acid phosphatase; TRAP). 0.5mls of cell suspension was added before being placed in the cell incubator for 2 hours. Following incubation, culture wells were washed twice in PBS and replaced with 1ml of osteoclast medium (25ng/ml M-CSF and 30ng/ml RANK-L). This step was performed to remove the majority of non-adherent cells and to leave adhered a relatively pure population of monocytes. Osteoclast growth medium was changed twice weekly between D1-14 with three medium changes in week 3 when cells were larger and more metabolic.

2.7 HISTOCHEMICAL STAINING TECHNIQUES

2.7.1 GIEMSA STAINING

Cells recovered from co-culture experiments were analysed morphologically. To prepare a cytospin, funnels were assembled with glass slides and adsorbent filter paper (Thermo Electron Co-operation, East Grinstead, UK). 100µl of cell suspension, typically $0.22x10^6$cells/ml, was pipetted into assembled funnels and centrifuged for 6 minutes at 400rpm (Shandon Cytospin© 2). Following this slides were air dried for a minimum of 2 hours before being fixed in pure methanol (Sigma Aldrich) for 10 minutes. Giemsa (Sigma Aldrich) was diluted 1:20 in distilled water and filtered. Samples were stained for 30 minutes in Giemsa solution prior to being rinsed and air-dried. This protocol was also used to stain recovered coverslips from co-culture experiments.

2.7.2 TARTRATE RESISTANT ACID PHOSPHATASE STAINING

A TRAP assay kit was obtained from Sigma Aldrich and performed in accordance to manufacturers' instructions. Briefly, ethanol-sterilised forceps were used to extract cover-slips harbouring adherent cells from D21 culture wells. Recovered cover-slips were washed in PBS before being fixed for 30 seconds in a mixture of citrate,

acetone and distilled water. Specimens were washed once in distilled water and allowed to air dry for at least 30 minutes prior to TRAP staining. Processed cover slips were placed in 24 well plates before the addition of pre-warmed (37ºC) tartrate solution that consisted of fast garnet, dissolved tartrate, napthol bi-phosphoric acid solution and acetate. Plates were left at 37 ºC for 1 hour in the dark. After thorough washing some specimens were counter-stained for 5 minutes with acid haematoxylin. Cover-slips were then rinsed thoroughly in distilled water and allowed to dry overnight before being mounted cell-side up on microscopic slides with DPX (Sigma Aldrich). Osteoclasts were defined as cells with 3 or more nuclei and exhibiting red/purple staining (i.e. exhibit tartrate resistance and able to convert napthol).

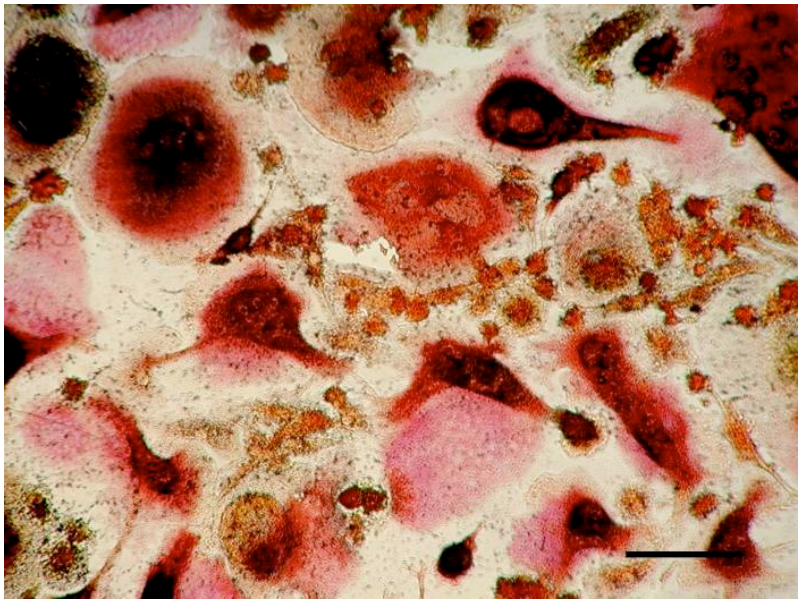


Figure 2-4. TRAP staining revealing large numbers of osteoclasts from a GD patient. Scale bar 50µm.

2.8 CELL VIABILITY ASSAYS

2.8.1 DRUG PREPARATION /STORAGE

All drugs were acquired from the Royal Free Hospital pharmacy department:-

(a) Melphalan (GlaxoSmithKline, Brentford, UK) was re-constituted in the supplied ethanol solvent to a stock concentration of 16.38mM. 200µl aliquots were stored at -20°C until required. Concentrations between 0-400µM were used in cytotoxic assays.

(b) Doxorubicin (Teva, Harlow, UK) was re-constituted in sterile water to create a stock concentration of 2mg/ml (3.4mM) which was stored at 4°C until use. Concentrations between 0-16µM were used in drug assays.
(c) Bortezomib (Jannsen-Cilag, Wycombe, UK) was dissolved and stored at -80°C in 15µl vials of 2µM solution. Concentrations between 0-160nM were used.
(d) Dexamethasone (Organon Labs Ltd, Cambridge, UK) is soluble in water and 15µl vials of 46.47mM stock solution were stored at -20°C until required. Cytotoxic assays used drug concentrations up to 10mM.
(e) U266 plasma cells were exposed to irradiation delivered by a GammaCell 3000 irradiator (Nodrion International Inc., Ottawa, Canada).

2.8.2 MTT DYE REDUCTION ASSAY

Cell lines or primary cells were pre-exposed to co-culture, doubling dilutions of drug or irradiation dose prior to performing the MTT dye reduction assay. This is a colorimetric test which is based on the mitochondrial reduction by living cells of the yellow tetrazole 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium into an insoluble purple formazan that is quantifiable by spectrophotometry (reference wavelength 540nm, measurement wavelength 650nm). The MTT assay is a measure of cell proliferation, viability or both. Typically, depending on experiment, 90µl of cell suspension ($1x10^4$-$1x10^5$ cells/well) was plated in triplicate in 96-well plates. 10µl of MTT reagent (Sigma Aldrich; 5mg/ml in PBS) was then added to each well and the plate was placed in a cell incubator for 2-4 hours. Plates were centrifuged at 1800rpm for 10 minutes and residual supernatant was carefully aspirated. Plates could be stored at -20°C at this stage prior to further analysis. DMSO, 100µL, was added to experimental wells followed by agitation for 15 minutes on a plate shaker set at 400rpm. Absorbance was quantified using a BMG FLUROstar Galaxy plate reader (MTX Lab Systems Inc., Virginia, US).

2.8.3 MTS DYE REDUCTION ASSAY

Cell lines or primary cells were pre-exposed to co-culture, doubling dilutions of drug or irradiation dose prior to MTS dye reduction assay. This is a colorimetric assay based on the same principle as the MTT assay. However the mitochondrial conversion of MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-

sulfophenyl)-2H-tetrazolium] by respiring cells has the advantage of producing a formazen product that is soluble in tissue culture. Therefore, this assay is useful for experiments involving adherent cells. Aliquots of MTS reagent were made up from supplied stock solutions and stored at -20°C in accordance to manufacturers' instructions (CellTiter 96® AQueous Non-Radioactive Proliferation Assay, Promega, Southampton, UK). Briefly, 20µl of MTS solution was added to each experimental well per 100µl of culture medium. Cells were placed in a cell incubator for between 1-4 hours before measuring absorbance at 490nm using a FLUROstar Galaxy plate reader (reference wavelength 650nm).

2.8.4 CHITOTRIOSIDASE ASSAY

The principle of the test is the release and fluorescent detection of 4-methylumbelliferone (4MU) on degradation of the substrate 4MU-chitotrioside by the plasma enzyme chitotriosidase (macrophage derived). This assay was performed on plasma samples collected from healthy controls or patients with Gaucher disease, myeloma or MGUS. This assay is based on the methodology of Hollak *et al.* (1994)[83].

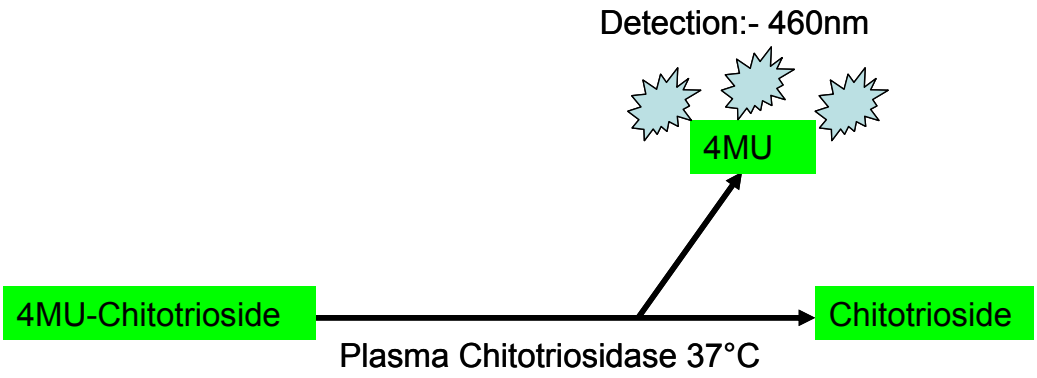


Figure 2-5. Principle of the chitotriosidase assay.
Residual chitotriosidase activity in sample plasma cleaves 4MU from 4MU-chitrotrioside and this is detected on a fluorimeter (460nm).

In duplicate sample wells, 5µl of patient plasma was added to 100µl of the substrate 4MU-chitotrioside (1mg dissolved in 57.8mls of McIlvaine Buffer [see section 2.8.5]; stored at -20ºC). Two wells, labelled Substrate Blank (SubB), were constructed in duplicate; these consisted of 5µl distilled water and 100µl of 4MU-chitotrioside. The plate was incubated for 1 hour at 37ºC. To each sample and substrate blank well, 1ml of 1M glycine solution (pH10.4) was added as a 'stopping-

agent' to terminate the enzymatic reaction. Two Standard Blank (StB) wells were created by adding 200µl of 4MU (176mg in 1 litre of distilled water) plus 0.9mls of 1M glycine solution. Analysis was then performed on a BMG-labs fluorimeter (excitation 365nm, emission 450nm). Chitotriosidase activity was calculated based on the following formula:-
(T-SubB)/(StaB-SubB)*(1000/5)*(1.105/1) = nmol/hr/ml plasma

T = Average of patients fluorometric reading
SubB =Average of Substrate Blank reading
StaB =Average of Standard Blank reading

Normal plasma activity is between 0-150nmol/hr/ml. Samples with chitotriosidase activity above maximal sensitivity of the fluorimeter were diluted or/and incubated for shorter incubation time.

2.8.5 BETA GLUCOSIDASE ASSAY

Recovered PBMCs were re-suspended in 2mls of Red blood cell (RBC) lysis solution (8.29g NH_4Cl [Sigma Aldrich]; 1g $KHCO_3$ [Sigma Aldrich]; 0.02g EDTA, di-sodium [Sigma Aldrich]; 1 litre distilled water) and left at room temperature for 5 minutes. As contaminating RBCs lead to artificially high results, this step was repeated if lysis was incomplete. PBMCs were washed twice in PBS (5 minutes; 300*g*), pelleted and stored at -80°C until analysis.

Prior to analysis, the pellet was thawed at room temperature (10 minutes) and re-suspended in 0.2-0.4mls of distilled water in order to create a cell lysate. The resulting suspension was vortexed and the supernatant was recovered after centrifugation at 1300rpm for 2 minutes. Total protein content was determined against a standard curve generated from dilutions of BSA between 0-1000 mg/ml. 25µl of the diluted standard or cell lysate was transferred, in duplicate, to a 96-well plate. 200µl of a 1:10 mixture of bicinchoninic acid (Sigma Aldrich) to copper (II) sulfate pentahydrate solution (Sigma Aldrich) was then added to each well. The immediate purple discoloration of sample wells indicated protein content above the top standard and the requirement for test sample dilution. The 96-well plate was

incubated for 30 minutes at 37°C before protein content was quantified by colorimetric measurement using a BMG FLUROstar Galaxy™ plate reader (absorbance wavelength 562nm; reference wavelength 650nm).

The following solutions were prepared in order to determine the enzyme activity of β-glucocerebrosidase:-

Buffer:-

McIlvaine Citrate Phosphate Buffer 0.15M pH5.2 (100mls) was prepared by mixing 46.9mls of 0.1M citric acid with 53.1mls of 0.2M $Na_2HPO_4.2H_2O$ solution.

Standard:-

A stock solution of 4MU was prepared by dissolving 176.9mg in 1L dH_20 and solubilisation was encouraged by warming to 80ºC (MW=176.9mg; Sigma Aldrich). 100µl of stock solution was added to 19.9mls of dH_20 to give a standard working solution of 5nmol/ml. Stock and working solutions were stored at -20ºC until required.

Stopping Reagent:-

100mls of 1M glycine (pH 10.4) solution was created by mixing the following:-
(1) 55.7mls of solution A (75g glycine [Sigma Aldrich] and 58g NaCl [Sigma Aldrich] in 1 litre of distilled water)
(2) 44.3mls of solution B (1M sodium hydroxide solution; Sigma Aldrich)
Stopping reagent was kept at room temperature until required.

4MU-Substrate/Inhibitor:-

0.2mmol/litre of 4MU-β-glucopyranoside (Sigma Aldrich) in McIlvaine buffer was mixed with 9.6g/l of the competitive enzyme inhibitor sodium taurocholate (Sigma Aldrich). The solution was stored at -20ºC until further use.

The enzymatic assay was then performed in a 24-well plate as described below:-

	Standard	Distilled water (Standard Blank)	Substrate /Inhibitor (Substrate Blank)	S1	S1
	“	“	“	S2	S2
	“	“	“	S3	S3

Table 2-2. Plate configuration of fluorometric based glucocerebrosidase assay.

15µl of sample supernatant was added to sample wells (S1, S2 etc.) and 150µl of 4MU-substrate/inhibitor solution was then added to Substrate Blank and sample wells. The plate was incubated at 37°C for 1 hour before termination of the enzymatic reaction by adding 1ml of 1M glycine stopping solution to sample and Substrate Blank wells. Following this, 15µl of sample supernatant was added to Standard, Standard Blank and Substrate Blank wells. In addition, 200µl of distilled water was added to Standard blank wells followed by 200µl of 4MU Standard into the corresponding standard well for each sample. Glycine stopping solution, 950µl, was added to Standard and Standard Blank wells to make a total volume of 1.165mls per well. Fluorometric measurement was performed using a BMG FLUROstar Galaxy™ plate reader (excitation 365nm, emission 450nm) and enzyme activity was calculated using the formula below:-

Enzyme activity = (Average of sample-Substrate Blank)/(Standard-Standard Blank)x(1000/protein mg/ml)*(1000/15)
(normal range 5.4-16.8 nmol/hr/mg protein)

2.9 WESTERN BLOT ANALYSIS

2.9.1 LYSATE PREPARATION

Harvested NCI-H929 plasma cells were centrifuged at 1000*g* for 5 minutes at 4°C before washing once in HBSS. Cells were re-suspended in 100µl of western blot lysis solution (containing protease and phosphatase inhibitors) before incubation on ice for 10 minutes. Following centrifugation at 14,000rpm for 15 minutes (4°C), the supernatant was recovered and stored at -80°C until analysis.

Western Blot Lysis solution:-20mM HEPES-KOH, 50mM NaCl, 2% nonyl phenoxylpolyethoxylethanol, 0.5% sodium deoxycholate, 0.2% sodium dodecyl sulphate, 1mM sodium orthovanadate, 1mM Ethylene glycol-bis-(2-aminoethyl)-N,N,N', N'-tetraacetic acid (EGTA) and10mM sodium fluoride. 10µl of protease inhibitor cocktail and10µl of 100mM phenylmethylsulfonyl fluoride was added to each 1ml of lysis solution. All reagents purchased from Sigma Aldrich.

2.9.2 PROTEIN QUANTIFICATION

Protein content of cell lysates was quantified using the Bio-Rad Protein assay (Bradford method). Doubling dilutions of BSA were used to create a standard curve (0 to 12µg/ml). 200µl of Bio-Rad protein assay solution (Bio-Rad, Birmingham, UK) was added to 800µl of test sample (1-4µl of lysate made up to 800µl with distilled water) or BSA standard. Following 5 minutes incubation at room temperature, samples were analysed by spectrophotometry at 595nm (NovaSpec II, Pharmacia). Protein content was ascertained from a standard curve correcting for background absorbance (sample blank) and sample dilution.

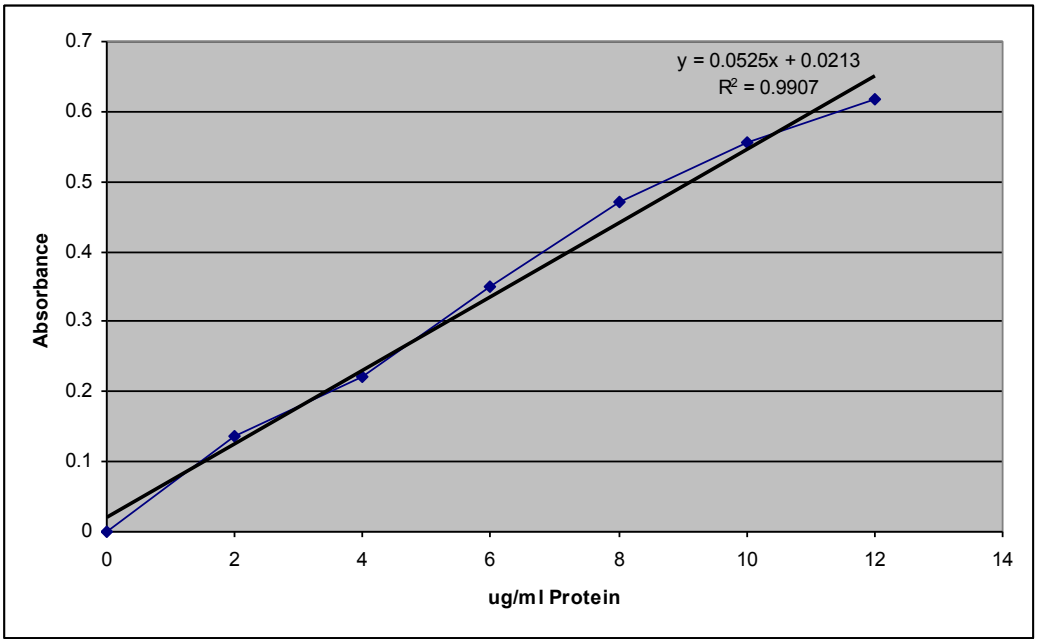


Figure 2-6. Bio-Rad Protein Assay. Standard Curve, absorbance versus protein content (µg/ml).

2.9.3 PREPARATION OF SAMPLES FOR LOADING ONTO GEL

Samples for analysis were diluted to give a total protein content of 30µg in 20µl lysis solution. Following this, 10µl of master mix (1:3 ratio dithiothreitol, Invitrogen: NuPage sample buffer, Invitrogen) was added to each sample. Samples were then centrifuged at 13,000rpm for 30 seconds, incubated at 80°C for 10 minutes and cooled for 5 minutes in tap water. A molecular weight ladder was created from a 1:3:2 ratio of HMW rainbow marker (GE Healthcare):lysate buffer:master mix. All samples were centrifuged at 13,000rpm for 30 seconds prior to loading.

2.9.4 GEL ELECTROPHORESIS

Gel tanks were filled with running buffer (50ml of NuPage running buffer, Invitrogen, to 950mls of distilled water). 12% tris-glycine gels (Invitrogen) were warmed to room temperature and rinsed in distilled water and running buffer. Samples were loaded using a Hamilton syringe. Anti-oxidant (Invitrogen, NP0005), 0.5mls, was added to the centre of the gel tank. Gel electrophoresis proceeded for 90 minutes using a Novex gel tank (200V, 50mA, 15W [Invitrogen]).

2.9.5 GEL TRANSFER

Transfer buffer consisting of 20mls of solution A (see below), 40ml methanol (Sigma Aldrich), 340ml distilled water and 400µl of anti-oxidant (Invitrogen, NP0005) was warmed to room temperature.

Solution A :- 20.5mM EDTA (Sigma Aldrich)
500mM Bicine (Sigma Aldrich)
500mM Bis(2-hydroxyethyl)-amino-tris(hydroxymethyl)-methane (Sigma Aldrich)

Following electrophoresis, the gel was removed and placed on 3mm thick filter paper. After wetting with transfer buffer, an appropriately sized nitrocellulose transfer membrane (HYBOND, GE Healthcare) was placed directly onto the gel before sandwiching with an additional piece of filter paper. Pads soaked in transfer buffer were added either side of the gel complex before being placed in a blotting cassette. Transfer proceeded for 90 minutes (25V, 125mA, 15.0W).

2.9.6 PONCEAU S STAINING

Following the transfer step, the membrane was stained for 2 minutes in Ponceau S stain (Sigma Aldrich) to confirm protein transfer. The membrane was then washed in 1x tris buffer containing 1% FCS and placed on a shaker for 2 minutes. Two additional washes in tris buffer were performed.

Tris Buffer x20 stock:-20mM tris(hydroxymethyl) aminomethane (Sigma Aldrich)
137mM NaCl
2g/l Tween-20 (Sigma Aldrich)
pH 7.4-7.8

2.9.7 PRIMARY ANTIBODY STAINING

FCS (0.5ml) and 100µl 10% azide (Sigma Aldrich) was added to 10mls of blocking solution (Tris Buffer containing 5% polyvinylpyrolidine; Sigma Aldrich). The relevant primary antibody was added before incubation of the membrane overnight at room temperature. All primary antibodies were used at a 1:1000 dilution.

Antibody	**Species**	**Manufacturer**	**kDa**
MCL-1	Rabbit	Santa Cruz Biotechnology	40
Bim	Rabbit	Santa Cruz Biotechnology	23, 15, 12
Bcl-xL	Rabbit	Cell Signalling	30
p53	Mouse	Santa Cruz Biotechnology	53
PUMA	Rabbit	Cell Signalling	23
Bcl-2	Mouse	Dako	26
BETA-ACTIN	Mouse	Sigma	45
ERK 1/2	Mouse	Cell Signalling	42, 44
p-ERK	Rabbit	Cell Signalling	140
PARP	Mouse	BD	116, 89

Table 2-3. Western blot antibodies.
Cell Signalling (New England Biolabs, Hitchin, Herts, UK). Santa Cruz Biotechnology (Heidelberg, Germany). DAKO (Stockport, UK).

2.9.8 SECONDARY ANTIBODY STAINING

Following incubation with primary antibody the membrane was washed with tris buffer for 45 minutes. Horseradish peroxidise (HRP) labelled secondary antibody (3µl anti goat or anti rabbit HRP; Dako) was then added to 10mls of blocking solution and 0.5ml FCS before incubating the membrane for a further 2 hours at room temperature. Membranes were washed three times in tris buffer.

2.9.9 VISUALISATION OF PROTEIN BANDS

Western Blots were developed with ECL (enhanced chemiluminscent) reagent (Fisher Scientific) according to the manufacturers' instruction and visualised on Fuji X-ray film.

2.10 DNA BASED TECHNIQUES

2.10.1 GAUCHER DNA ANALYSIS (STRIPTEST)

Gaucher DNA strip test kits were obtained from ViennaLabs (Vienna, Austria) and performed in accordance with the manufacturers' instructions. Briefly, blood was collected from consented individuals into EDTA tubes and aliquots of 500-1000µl were frozen at -80°C until required. Pre-prepared lysis and DNA binding solutions were used to isolate DNA from whole blood via several different centrifugation steps. Processed DNA was cooled on ice for immediate use. Alternatively, isolated DNA was stored refrigerated for up to 1 week at 4°C or frozen at -80°C until required.

Processed DNA was amplified using Taq Polymerase (0.2µl; Applied Biosystems; Warrington, UK) in 2 different amplification mixtures covering the 10 wild type and mutated Gaucher alleles. PCR was performed in accordance with specifications provided on a Bio-Rad S1000™ thermal cycler (Pre-PCR 94°C/2minutes; PCR 35 cycles; 94°C/15 seconds, 58°C/30 seconds, 72°C seconds; final extension 72°C/3 minutes).

Following amplification, products were washed, mixed with hybridisation solution and treated with denaturing solution. The products were then incubated with test

strips in the dark at 45°C for 30 minutes. Test strips then were treated with conjugate solution and colour developer prior to reading. Test strips were impregnated with the oligonucleotides of the 10 common mutations found in Jewish patients with GD (84GG, IVS2+1, N370S, V394L, D409H, L444P, R463C, R496H, Rec TL and RecNciI).

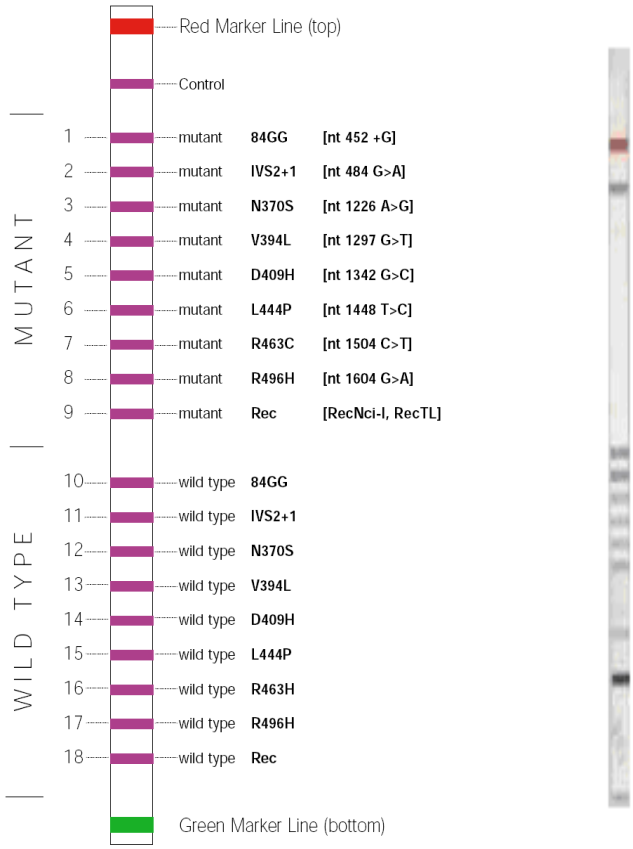


Figure 2-7. Gaucher Strip Test (ViennaLabs).

2.10.2 DNA ISOLATION

DNA was extracted from either freshly collected peripheral blood (EDTA sample) or from stored whole blood samples (-80°C). Thawed samples were diluted with PBS (6mls total) and pipetted gently onto 5mls of Ficoll-Pague™-Plus before being centrifuged at 2100rpm for 15 minutes (brake off). The isolated buffy coat was washed in PBS before centrifugation at 400*g* for 5 minutes. From here on, the method of DNA extraction was identical for both freshly acquired and frozen samples. Samples were incubated at room temperature with RBC lysis solution (8.29g NH_4Cl; 1g $KHCO_3$; 0.02g EDTA, di-sodium; 1 litre distilled water) for 5

minutes, centrifuged, re-suspended and washed again in PBS. Cells were thoroughly re-suspended in 2mls of whole cell lysis solution (Qiagen, Crawley, UK). An appropriate volume of protein precipitation solution (Qiagen) was then added (whole cell lysis solution: protein precipitation solution 3:1 v/v). Samples were vortexed for 30 seconds and centrifuged at 2500rpm for 10 minutes. Soluble DNA from the resulting supernatant was removed using a pastette and precipitated from solution by the addition of an equal volume of isopropanol.

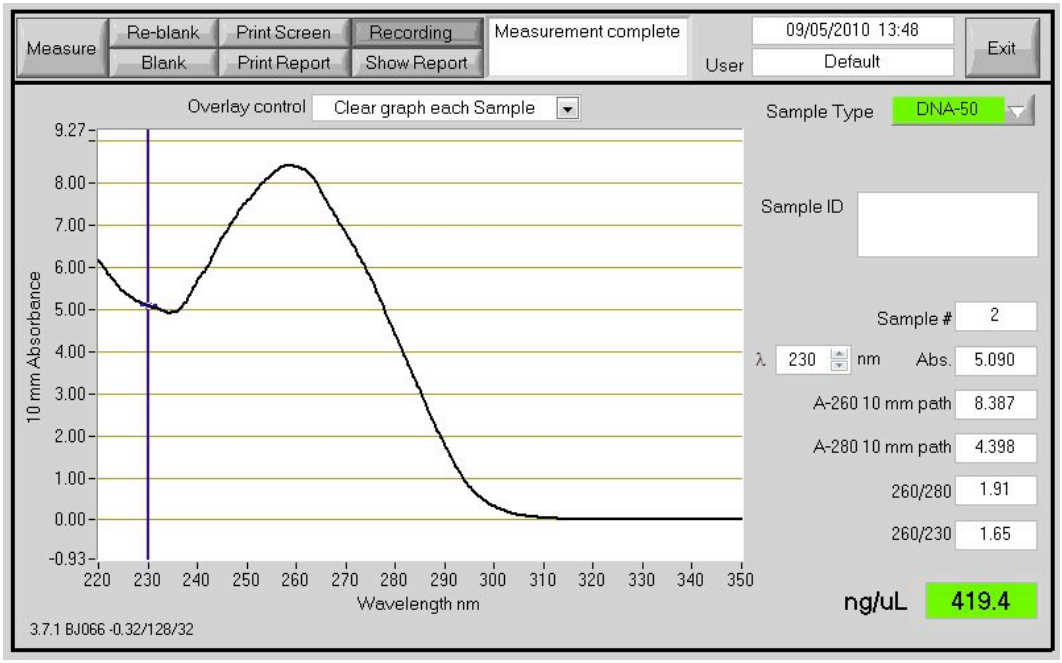


Figure 2-8. Nanodrop quantification.
Processed sample was analysed for DNA quantity and evidence of RNA or protein contamination.

Samples were centrifuged for 5 minutes at 3000rpm, the supernatant discarded, and the remaining DNA pellet was dried at room temperature for 2 hours before dissolving in 50-200µl of distilled water. DNA was rehydrated for a minimum of 24 hours prior to determining DNA concentration and quality (Nanodrop 1000™, spectrophotometer, Thermo Scientific, Wilmington, USA). Protein/RNA contamination was assessed by the 260/280 ratio with a value of 1.8-2.1 being desirable.

2.10.3 POLYMERASE CHAIN REACTION (PCR) SEQUENCING

Exons 5-11 of the *GBA1* gene were analysed by direct DNA sequencing. Exon blocks 5-7 and 8-11 were expanded in two separate PCR reactions using a pair of intronic primers specific for the *GBA1* gene and not the pseudogene. Following initial amplification, the products were subjected to a second PCR reaction using an exon-specific pair of intronic primers. Primer sequences and PCR reaction conditions were performed as described by Stone *et al*. (2000)[49]. In addition, the direct amplification and sequencing of exon 6 was performed in a subset of samples.

(A) Initial Amplification Reaction

Each PCR reaction contained 5µl of diluted DNA (20ng/µl) and 20µl of a master mix Table 2-4) containing the appropriate dilution of primers (exons block 5-7, 8-11; Table 2-5). Master-mix (20µl) was aliquoted into 96-well plates (Appleton Woods, Birmingham, UK) using an automatic pipette (Anachem, Luton, UK) before the addition of diluted DNA. The PCR plate was sealed with an adhesive plate sealer (Platemax™, Axygen, California USA; Distributed VWR, Leicestershire, UK) before being centrifuged at 1000rpm to ensure adequate mixing and the eradication of air bubbles. PCR reactions were performed as detailed in Table 2-6 (PCR-blocks, C1000™ thermal cycler, Bio-Rad Laboratories, Hemel Hampstead, UK). All reagents were defrosted and placed on ice prior to use.

Reagent	µl/Sample	Company
X10 PCR buffer II	2.5	Applied Biosytems Limited, Warrington, UK
$MgCl_2$ – 25mM	1.5	Applied Biosytems Limited, Warrington, UK
dNTPs – 2mM	2.5	GE Healthcare, Chalfont St Giles, UK
Forward Primer 10mM	1.0	Invitrogen, Paisley, UK
Reverse Primer 10mM	1.0	Invitrogen, Paisley, UK
AmpliTaq Gold DNA Polymerase 5u/ml	0.25	Applied Biosytems Limited, Warrington, UK
Distilled Water	11.25	-

Table 2-4. Constituents of the PCR master mix solution.

GBA	Forward Sequence	Reverse Sequence
Exons 5-7	5'-GACCTCAAATGATATACCTG-3'	5'-AGTTTGGGAGCCAGTCATTT-3'
Exons 8-11	5'-TGTGTGCAAGGTCCAGGATCAG-3'	5'-CCACCTAGAGGGGAAAGTG-3'

Table 2-5. Exon block primers for the initial PCR reaction.

PCR Reaction	Conditions
Exons 8-11 Amplification	95°C – 15 minutes 34 cycles (95°C 1 minute, 61°C 1 minute, 72°C 1:30 minutes) 72°C – 5 minutes 4°C – Indefinitely
Exons 5-7 Amplification	95°C – 15 minutes 34 cycles (95°C 1 minute, 58.5°C 1 minute, 72°C 2 minutes) 72°C – 5 minutes 72°C – 5 minutes 4°C – Indefinitely
Sequence Reaction	24 cycles (96°C 1 minute, 50°C 5 seconds, 60°C 4:00 minutes) 4°C – Indefinitely
ExoSap/Enzyme digest	37°C – 30 minutes 80°C – 15 minutes 4°C – Indefinitely

Table 2-6. Thermal cycler programs for DNA expansion/sequencing.

(B). Sequence Reaction

Following initial amplification, DNA products were purified by adding 15µl of sample to 6µl ExoSap-IT™ (GE Healthcare; Stored -20°C) in 96-well plates. After sealing, the microplate was centrifuged briefly at 100rpm to eradicate air bubbles. ExoSap-IT™ contains two hydrolytic enzymes that degrade excess deoxynucleoside triphosphates (DNTPs) and residual single stranded primers. Plates were incubated at 37°C for 30 minutes (Bio-Rad) before inactivating the ExoSap-IT™ by heating to 80°C for 15 minutes.

Sequencing was performed using the BigDye™ v1.1 platform (Applied Biosystems Limited). Details of the sequencing primers are given in Table 2-7. A master mix containing 0.5µl BigDye™, 2µl BigDye™ x5 sequencing buffer and 5.5µl distilled water per sample was placed on ice. Each reaction well contained 8µl master mix, 1µl PCR product and 1µl of 0.5mM primer (Forward or Reverse). Sequence primers were manufactured by Invitrogen and reactions were performed in duplicate in both

directions (forward and reverse). Prior to product amplification on the Bio-Rad, the microplate was centrifuged at 300*g* (Table 2-6). Following amplification, products were purified by centrifugation (1000*g*, 3 minutes) through a DyeEx^{TM} plate. The DyeEx^{TM} plate utilises gel-filtration technology to remove untagged dye terminators in order to obtain a 'cleaner' sequence. DyeEx^{TM} plates were used in accordance with the manufacturers' instructions (Applied Biosytems). The microplate was incubated for 60 minutes at 72°C in order to evaporate excess fluid. Dried product was re-suspended in 10µl of HI-DI formamide (Applied Biosytems Limited) and briefly vortexed. Reactions were transferred to a sequencing micro-plate (MicroAmp, Applied Biosystems), analysed immediately or stored frozen until required at -20°C. Sequences were acquired using a 24 capillary ABI C3500 DNA sequencer (Applied Biosytems).

GBA Exon	Forward Sequence	Reverse Sequence
5	5-GCAAGTGATAAGCAGAGTCC-3	5-AGCAGACCTACCCTACAGTTT-3
6	5-CTCTGGGTGCTTCTCTCTTC-3	5-ACAGATCAGCATGGCTAAAT-3
7	5-TTGGCCGGATCATTCATGAC-3	5-AGTTTGGGAGCCAGTCATTT-3
8	5-TGTGTGCAAGGTCCAGGATCAG-3	5-TTTGCAGGAAGGGAGACTGG-3
9	5-CACAGGGCTGACCTACCCAC-3	5-GCTCCCTCGTGGTGTAGAGT-3
10	5-CAGGAGTTATGGGGTGGGTC-3	5-GAGGCACATCCTTAGAGGAG-3
11	5-GTGGGCTGAAGACAGCGTTGG-3	5-ACCACCTAGAGGGGAAAGTG-3

Table 2-7. Forward and reverse sequencing primers for GBA exons 5-11.

2.10.4 AMPLIFICATION/SEQUENCING OF EXON 6

Following amplification of exon block 5-7, sequencing of exon 6 failed in a few samples due to sample degradation, despite using alternative primers. This occurred mainly in archived whole blood samples that were frozen at -80°C for up to 2 years in duration. This was overcome by using exon 6 primers for both the initial amplification and sequencing reactions. The reverse primer detailed in Table 2-7 was specific for the *GBA1* gene and not the downstream *GBAP* pseudogene (compared to NCBI DNA Pseudogene Sequences AF032368.1, J03060.1 and AF267177).

Using 10mM dilutions of the forward and reverse primers (exon 6) samples were initially amplified using the same master mix described in Table 2-4. A temperature gradient was performed (Figure 2-9) to optimize the PCR gradient and products were subjected to gel electrophoresis (2% agarose; Sigma Aldrich). Based on these results, an annealing temperature of 60.9°C was used in the final PCR program which is described below:-

95°C 15 minutes,

34x (95°C 30 seconds, 60.9°C 40 seconds, 72°C 1 minute)

72°C 5 minutes

Cooling to 4.0°C.

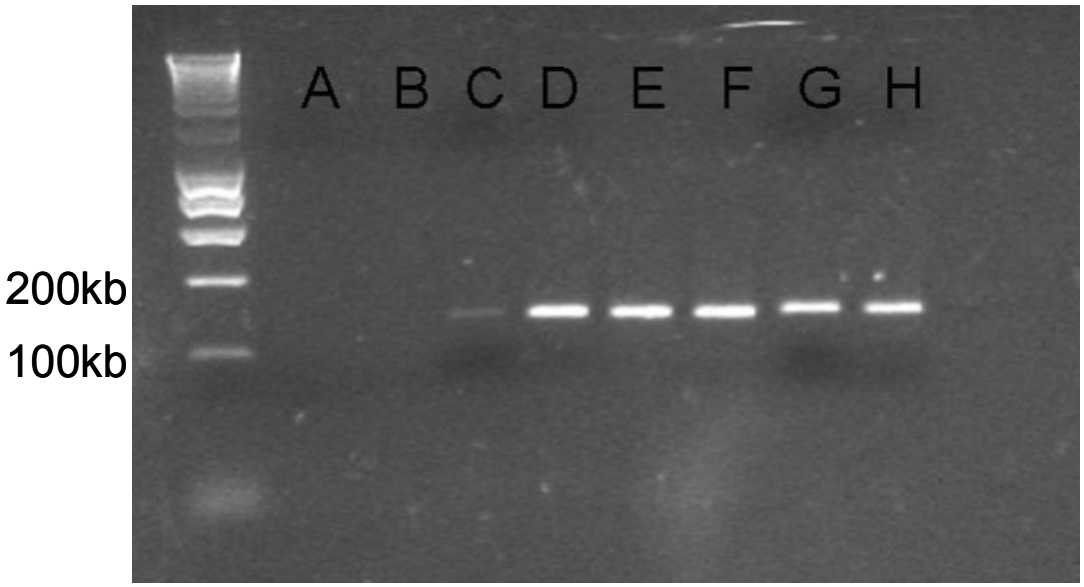


Figure 2-9. Temperature gradient determination for Exon 6.
Lane A (55.0°C), B (56.0°C), C (58.0°C), D (60.9°C), E (64.5°C), F (67.5°C), G (69.2°C) and H (70.0°C).

The sequencing reaction was performed as described above using the same primer set as the initial amplification reaction.

2.10.5 SEQUENCE ANALYSIS

Analysis of acquired sequences was performed using Mutation Surveyor (BioGene Ltd, Cambridgeshire, UK). All mutations were confirmed on both forward and reverse sequences. A reference sample of the *GBA1* gene was obtained from the NCBI DNA Sequence Database.

2.10.6 84GG/IVS2+1 MUTATIONS

Exon 2 harbours the 84GG and the IVS2+1 mutations. Wild or mutant forward primers were used, together with a reverse primer specific for the *GBA1* gene but not the pseudogene (Mistry *et al*. 1992). A master mix was created as detailed in Table 2-8.

Reagent	µl/sample	Primer Sequences
Distilled water	22.5	
$MgCl_2$ 25mM	3	
2mM DNTPS	5	
X10 PCR Buffer II	5	
Forward Primer (Wild or mutant) 10mM	2	**WILD** 5 - GCATCATGGCTGGCAGCCTCACAGGACTGC - 3 **MUTANT** 5 – GCATCATGGCTGGCAGCCTCACAGGACTGG – 3 Invitrogen, Paisley, UK
Reverse Primer	2	5 – GCCCAGGCAACAGAGTAAGACTCTGTTTCA – 3 Invitrogen, Paisley, UK
AmpliTaq Gold DNA Polymerase 5u/ml	0.5	

Table 2-8. 84GG sequence reaction.

To 40µl of master mix 10µl of diluted DNA was added (20ng/µl). DNA was amplified using the following PCR program:-

95°C 15 minutes
34x (95°C 45 seconds, 68°C 45 seconds, 72°C 45 seconds)
72°C 5 minutes
Cooling to 4.0°C

Following amplification, products were subjected to gel electrophoresis on a 2% agarose gel and bands were visualised with Syber Safe® (Invitrogen) by trans-illumination. The desired product was 255bp in length. Inclusion of a known 84GG heterozygote within the terminal lane acted as an internal control (Figure 2-10). The IVS2+1 mutation was identified by enzyme digest from the same 255bp product (wild forward primer) amplified for the detection of the 84GG allele. The IVS2+1 mutation leads to disruption of an HphI restriction digest site in the expanded

product. Therefore 3 bands are visualised in those with unmutated GBA alleles (41bp, 72bp and 141bp), 4 in heterozygotes (41bp, 72bp, 141bp and 213bp) and 2 in homozygotes (213bp and 42bp). To 20µl of amplified product, 2µl of HphI enzyme (NEB, Hitchin, UK), 3µl NEB4 buffer (NEB) and 5µl of distilled water was added. Samples were incubated at 37°C for 2 hours on a Bio-Rad thermal cycler prior to gel electrophoresis on a 3% agarose gel (Figure 2-11).

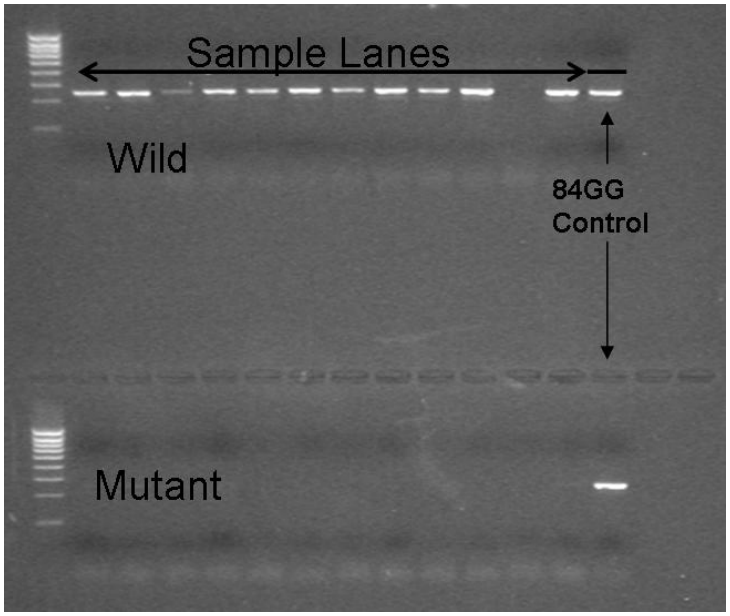


Figure 2-10. Gel electrophoresis, 84GG mutation.
Amplified products were analysed on a 2% agarose gel with the terminal lane containing a sample from a known heterozygote.

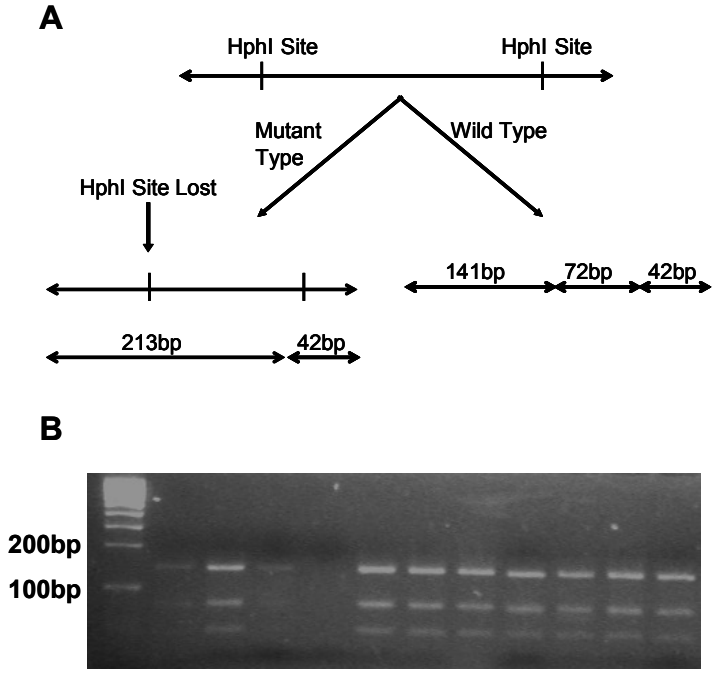


Figure 2-11. IVS2+1 Mutation. Amplified products were analysed on a 3% agarose gel.

2.11 CELLULAR KILLING ASSAYS

2.11.1 NK-T KILLING ASSAY

NK-T cells were isolated by magnetic column technology from healthy controls or patients with GD (effector cells) before co-incubation in a cytotoxicity assay with various target cells (K562, NCI-H929 or JJN3 cell lines).

Peripheral blood (40mls) was diluted 1:2 with PBS and layered over 20mls of Ficoll-Pague™-Plus before centrifugation at 650*g* for 25 minutes (4°C; brake off). Recovered cells were washed twice in PBS (400*g*, 15 minutes) and re-suspended in 10mls of Magnetic-Activated Cell Sorting buffer (MACS buffer; PBS, 0.5% BSA, 2mM EDTA). MACs buffer was kept on ice until use. Prior to NK-sorting, flow cytometry was performed for CD3/56 quantification. The remaining cells were counted, centrifuged and re-suspended in 80μl MACS buffer/10^7 cells. 20μl of CD56 micro-bead solution was added (Miltenyl-Biotec, Surrey, UK) per 10^7 mononuclear cells. Samples were incubated for 15 minutes at 4°C.

The LS (large-size) separation column (Miltenyl Biotec) was placed in a VarioMACs magnet/stand and primed for use by flushing with 5mls MACs buffer. Following incubation, samples were diluted with 5mls of MACs buffer and gently pipetted into the separation column in order to minimise air bubbles. The LS column was flushed 3 times with MACs buffer (3mls) in order to elute the CD56-ve fraction. CD56+ve cells were collected after removing the LS column from the magnet and flushed with 5mls MACs buffer using the provided plunger. Recovered cells (+ve and –ve fraction) were centrifuged for 10 minutes at 400*g* before re-suspension in at $2x10^6$ cells/ml in R10 medium. Typically, high purities (>95%) of CD56+ve cells were isolated. Aliquots of CD56+ve and CD56-ve cells were incubated alone or supplemented with 200IU/ml (Miltenyl Biotec) of human IL-2 overnight (approximately 16 hours).

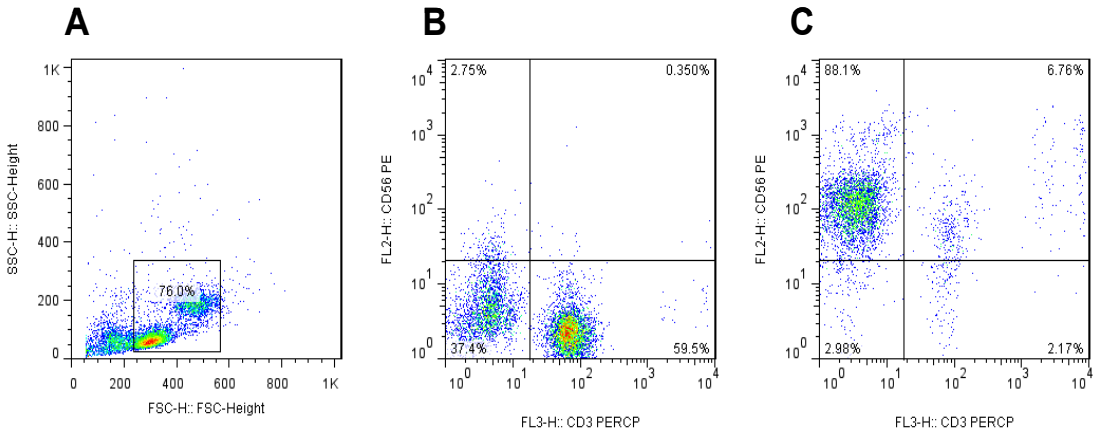


Figure 2-12. Flow cytometry demonstrating (A) Pre-sorted cells, (B) CD56-ve cells (C) CD56+ve cells post magnetic sorting.

All target cell lines (K562, NCI-H9292 and JJN3) underwent growth medium change together with viability testing (trypan blue) the day before co-culture. Cell line viability was maintained at greater than 90%. Target cells were stained with the red membrane dye PKH26 (Sigma Aldrich) according to the manufacturers' instructions. Briefly, target cells were re-suspended in 500µl of diluent C (Sigma Aldrich) at $1x10^6$cells/ml after washing twice in PBS. Following re-suspension, 500µl of diluted dye (498µl Diluent C, 2µl PKH26 ethanolic dye solution) was added to target cells and samples were incubated at room temperature for 3 minutes. Staining was halted by the addition of 1ml FCS and to this, 5mls of R10 medium, was added 1 minute later. Cells were centrifuged and re-suspended in 2mls of growth medium. Successful membrane staining was confirmed by flow cytometry (PKH26 fluorescent in FL2 channel). PKH26 staining is toxic to cells and background death was quantified by positivity for the nuclear stain TO-PRO®-3 iodide.

Killing assays (CD56+ve and CD56-ve fractions) were performed at an effector:target ratio of 5:1 ($2x10^5$cells: $4x10^4$ cells). After mixing, samples were placed in a cell incubator for 4 hours prior to analysis. Killed target cells were identified by dual positivity for PKH26 and TO-PRO®-3 iodide. Adjustment was made for background cell death secondary to staining.

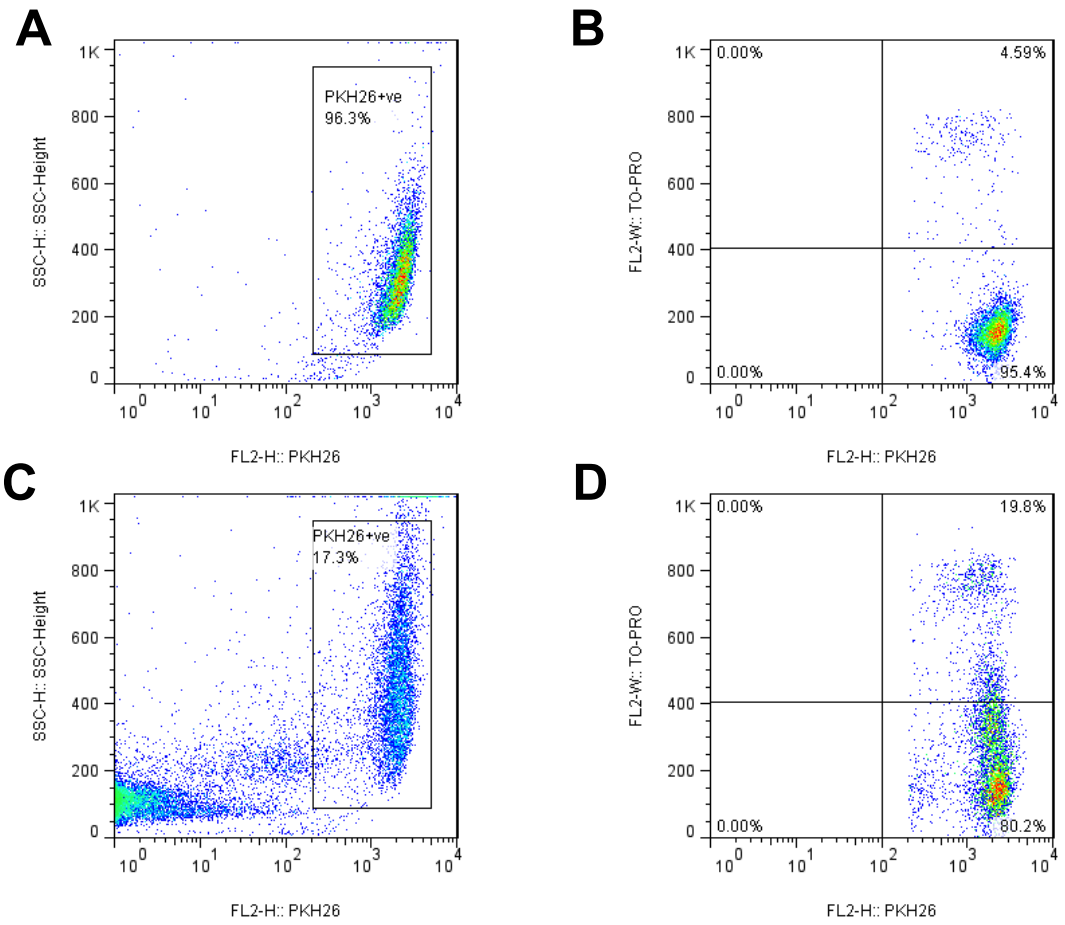


Figure 2-13. CD56+ve effector cell killing assays.
Flow cytometry demonstrating (A, B) PHK26 stained target cells and background cell death as indicated by the % of apoptotic TO-PRO+ve cells in the right upper quadrant. (C, D) NK killing assay of JJN3 cells.

2.11.2 PBMC KILLING ASSAYS

PBMC were isolated from peripheral blood as described above. PBMCs were incubated with 200IU/ml of IL-2 overnight. Killing assays were performed at different effector:target ratios (40:1, 20:1 and 10:1) as described in the section above with $4x10^4$ K562, NCI-H929 or JJN3 PKH26 stained cells.

2.12 FLUORESCENT MICROSCOPY

2.12.1 BODIPY-LACTOSYLCERAMIDE/LYSOTRACKER STAINING

Cytospins were made of isolated PBMCs ($2x10^4$ cells, 400rpm and 3 minutes). Using a water indelible pen, a circle was drawn around the cells. Approximately 200µl of

5μM BODIPY-lactosylceramide (Molecular Probes; Supplied Invitrogen) was pipetted over the cells before incubating in the dark for 60 minutes. After washing three times for 1 minute in R10 medium, specimens were incubated in the dark for a further 170 minutes. R10 was then replaced with 200μl of 200nM lysotracker solution (Invitrogen) for an additional 30 minutes. Following this, specimens were washed twice in PBS before fixing with 4% paraformaldehyde in the dark for 20 minutes. Specimens were washed twice in PBS (5 minutes) before mounting a glass coverslip (Prolong®Gold anti-fade reagent, Invitrogen). Specimens were allowed to dry overnight before analysis. Samples were analysed using a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss Ltd., Welwyn Garden City, UK).

2.13 STATISTICS

All experimental data were analysed using GraphPad Prism 5.0™ (GraphPad Software Inc., California, USA) or Microsoft Excel 2003 (Microsoft Co-operation, California, USA). Standard deviation (SD), mean, median and inter-quartile range were used to summarise data. The D'Agostino & Pearson omnibus normality test was used to identify whether the data had a Gaussian distribution. A paired t-test and Wilcoxon signed rank test were used for paired parametric and non-parametric data respectively. An unpaired t-test was used to compare parametric data, whereas non-parametric data were analysed using a Mann-Whitney test. One-way ANOVA (analysis of variance) was used to compare the means of 3 or more discrete populations. A p value <0.05 was considered to be statistical significant. Drug activity was summarised to an IC_{50}, which was calculated using a sigmoidal concentration-response model with a variable slope. Data correlation was performed using a Spearmen test. All statistical tests were two-tailed, unless indicated.

3 PATIENT DEMOGRAPHICS AND GBA MUTATIONS

3.1 INTRODUCTION

3.1.1 GAUCHER DISEASE AND GAMMOPATHY

Patients with GD have an increased incidence of MGUS, polyclonal gammopathy and multiple myeloma[81;130;139], as described in section 1.3.2. Polyclonal gammopathy is common in GD being more frequent in untreated patients[43] and reported in 14-64% of untreated patients with type I GD[43;74;138;162]. In an untreated paediatric population with a mean age of 8.5 years, 21/23 (91%) had evidence of polyclonal gammopathy[234]. One of the first descriptions of monoclonal gammopathy in patients with GD came from Pratt *et al.*, in 1968, in which 4/16 patients had an IgG M-band. Studies from America, Germany and the Netherlands have reported a 9-25% incidence of MGUS in patients with GD[81;138;139]. Data from a Gaucher Registry identified 10 out of the 2,742 patients to have multiple myeloma, conferring an estimated relative risk of approximately six times[130]. Data from an Israel based registry reported 2/500 patients with type I GD to have multiple myeloma, and concluded an increased risk[129].

In a small cohort of 63 patients (13/63 paraprotein), de Fost *et al.* (2008) failed to establish baseline disease severity as a risk factor for paraproteinaemia[81]. Although not statistically significant, GD patients with a paraprotein, in this study, were found to have a higher baseline Zimran severity score (ZSS) and chitotriosidase activity those without an M-band. Brautbar *et al.* (2004) failed to demonstrate an association between disease severity and the presence of polyclonal gammopathy[43], but visceral disease was suggested to be risk factor for gammopathy in one study[138].

Although, there have been several studies detailing the incidence of gammopathy in GD patients, several questions remain unanswered. Based on the low number of published reports it is not possible at present to dismiss disease severity as a risk factor for gammopathy. Chitotriosidase activity is a sensitive marker of disease activity[83] but due to the presence of null mutations (33% heterozygotic and 6% homozygotic)[357]

comparative analysis may fail to identify a true difference when comparing small cohorts of individuals. Macrophage derived biomarkers including S-ACE and acid-phosphatase have yet to be compared between individuals with gammopathy and those with normal immunoglobulins. Such an association would add weight to hypotheses exploring the ability of lipid-laden macrophages to support plasma cell growth and survival. Furthermore, it is currently unknown, whether residual glucocerebrosidase activity, is predictive of gammopathy. In addition, no study has addressed, whether individuals with monoclonal gammopathy have more severe disease than those with polyclonal gammopathy and whether markers of disease severity predict for the presence of gammopathy in the era of ERT. These lines of investigation are addressed below.

3.1.2 MACROPHAGE PATHOLOGY – COMMON TO MYELOMA AND GAUCHER DISEASE

GD is caused by low glucocerebrosidase activity[23] and is associated with the accumulation of lipid laden macrophages or Gaucher cells. Gaucher cells are larger than normal macrophages with a wrinkled 'onion-skin' appearance to their cytoplasm. Foamy macrophages (Pseudo-Gaucher cells), similar to Gaucher cells, are seen in the bone marrow of patients with haematological cancer including chronic myeloid leukaemia[280] and myeloma[358]. Macrophages are more abundant in the bone marrow of those with multiple myeloma than controls[245]. These histological findings have lead to the suggestion that macrophage abnormalities are common to both GD and non-GD patients with plasma cell disorders. Investigators have postulated that Gaucher and Pseudo-gaucher cells, via similar pathological mechanisms, predispose to a cancerous microenvironment. The accumulation of Pseudo-Gaucher cells in those with haematological cancer may pre-date the development of malignancy. Alternatively, Pseudo-Gaucher cells may be innocent bystander cells, which develop as a secondary consequence of haematological cancer. Non-GD patients with malignancy have been hypothesised to acquire abnormalities in glucosylceramide metabolism. Zidar *et al.* (1987) reported the accumulation of pseudo-Gaucher cells in a patient with Hodgkin's lymphoma[278]. The authors went on to confirm normal leucocyte glucocerebrosidase activity in Pseudo-Gaucher cells. However, this finding does not exclude saturated

glucocerebrosidase activity, secondary to excessive cell breakdown, leading to the development of a similar macrophage phenotype to that seen in GD[278].

Kampine *et al.* (1967) hypothesised that patients with haematological cancer have acquired abnormalities in sphingolipid metabolism within the cancerous clone, in comparison to the discussion above relating to tissue macrophages[277]. However, based on the hydrolysis of ^{14}C-labelled substrates, enzyme activity, including that of glucocerebrosidase, was not reduced in leucocytes of patients with either acute or chronic leukaemia (myeloid or lymphoid)[277]. Glucocerebrosidase hydrolyses glucosylceramide into glucose and ceramide. Intracellular ceramide has a role in the regulation of cell cycling, the immune system and the induction of apoptosis within cells, normal or cancerous.[20]

Boven *et al.* (2004), reporting spleen histology, showed that Gaucher cells have the phenotype of alternatively activated (M2) macrophages. Tumour infiltration by M2 macrophages are linked to a poorer outcome in cancer patients[268]. Speculatively, acquired glucocerebrosidase deficiency, secondary to a neoplastic process, could lead to the accumulation of M2 macrophages in non-GD patients. Abnormalities within the bone marrow microenvironment contribute to the emergence, malignant transformation, growth characteristics and chemo-resistance of plasma cell clones[359;360]. The demonstration of low glucocerebrosidase activity in macrophage precursors of non-GD individuals with plasma cell dyscrasias would support chapter hypotheses suggesting common aetiology to that seen in GD.

Chitotriosidase is a macrophage-derived enzyme detectable in plasma, which was initially described based on its ability to hydrolyse artificial chitotrioside substrates[357]. Chitotriosidase is synthesised by activated macrophages but its role in humans is unclear[90]. Macrophage storage is the hallmark of GD and Gaucher cells secrete large quantities of chitotriosidase. Plasma chitotriosidase activity correlates with both disease severity and the success of ERT in GD[83;361]. Chitotriosidase activity is elevated in the plasma of individuals with inflammation, sarcoidosis[362], atherosclerosis[363],

thalassaemia[364], advanced prostate cancer[365], malaria[366] and other lysosomal storage disorders[90;96;367;368] (fucosidosis, Niemann Pick disease, Fabry disease and galactosialidosis). However, in comparison, the level of increase in plasma enzyme activity in GD is pronounced. Normal chitotriosidase activity, determined by the method of Hollak *et al.* (1994)[83], using a fluorometric enzyme assay with a 4-methyl umbelliferone (4-MU) substrate, is typically less than 200 nmol/mL/hr. However, chitotriosidase activity is usually greater than 10,000nmol/mL/hr in untreated patients with GD and can be >100,000nmol/mL/hr.[369]. It is not reported in the literature, whether non-GD individuals with plasma cell disorders, including multiple myeloma, have increased chitotriosidase activity, therefore sharing similar macrophage pathology to patients with GD.

The chitotriosidase gene (*CHIT1*) is located on chromosome 1q31-32, is approximately 20 kilobases in length and contains 12 exons. One third of the population are heterozygous for a 24bp duplication that inactivates the enzyme. Approximately 6% of the population have homozygous null mutations within the chitotriosidase gene. The frequency of null mutations, either homozygous or heterozygous, was found to be similar in a Dutch based study between the general population and Ashkenazi Jews[357]. *GBA1*, the gene coding β-glucocerebrosidase, has been mapped to chromosome 1q21 and is approximately 50,000kb apart from the *CHIT1* gene (http://www.ncbi.nlm.nih.gov/gene). Regarding the possibility of gene linkage, these loci are separated by a base pair distance equivalent to the size of the Y-chromosome[370].

3.1.3 GBA GENE AND PSEUDOGENE

The *GBA1* gene is located to chromosome 1q21 and codes for 11 exons and 10 introns (see chapter 1.2.1) [27]. About 300 mutant alleles have been reported and 10 alleles complete the genotype in approximately 90% of Ashkenazi Jews with GD (84GG, IVS2+1, N370S, V394L, D409H, L444P, R463C, R496H, RecNciI and RecTL)[27;36]. The *GBA1* pseudogene (*GBAP*) is closely located to the *GBA1* gene and shares 96% sequence homology[27]. Therefore, the construct of primers, for the screening of *GBA1* mutations in GD is crucial, in order to avoid amplification of the *GBAP* locus. The

majority of reported mutations are found in exons 5-11 with no disease causing alleles being described in exon 1[27]. As discussed in section 1.2.2, the N370S and L444P alleles are the commonest encountered mutations.

3.1.3.1 PARKINSONS DISEASE – *GBA1* MUTATIONS

Patients with GD are more likely to develop Parkinson's disease (PD). In a cohort of 57 patients of mixed ethnicity with PD, *GBA1* mutations were found in brain samples of 12 patients (21%) at post mortem[371]. Four individuals were found to have polymorphisms, including E326K, 8 had disease-associated mutations with 10/12 patients being carriers for a *GBA1* mutation. In addition, within the last decade, the frequency of *GBA1* mutations has been found to be increased in populations of PD patients, irrespective of ethnicity[352]. Aharon-Pertz *et al*. (2007) screened Ashkenazi Jews, including 1543 healthy controls and 99 patients with PD, for 6 common *GBA1* mutations (N370S, L444P, 84GG, IVS2+1, V394L and R496H). They found a higher frequency of *GBA1* mutations in the PD cohort (mostly heterozygotes, 3 homozygotes), compared to healthy controls (31.3%v6.2%)[372]. Geographical location dictates which *GBA1* mutations are found in individuals with PD. In the UK, N370S and L444P were the commonest mutations[373], whereas R120W and RecNciI were the most frequent in patients from Japan[374]. *GBA1* polymorphisms, including E326K and T369M, are not uncommon in those with familial PD, being associated with a low age at onset[375].

Several theories have been postulated as to why carriers of *GBA1* mutations have an elevated risk of PD. Historically, leucocyte glucocerebrosidase activity, depending on methodology, is unable to reliably distinguish between heterozygotes and those with GD[353]. Therefore, carriers may have some impairment in sphingolipid metabolism, which confers an elevated risk to PD, similar to that observed in those with GD.

Under normal circumstances, α-synuclein, a soluble protein found in brain tissue, is complexed to lipids including within the cell membrane. However, in certain neurological diseases, including PD and Lewy Body disease, α-synuclein can aggregate to form β-pleated sheets consisting of insoluble amyloid fibrils. Decreased

glucocerebrosidase activity has been suggested to promote a cellular environment favourable to fibril deposition and neuro-degeneration[352]. Using CBE to inhibit glucocerebrosidase, investigators have demonstrated the accumulation of α-synuclein in the midbrain of C57BL/6 mice and a cultured neuroblastoma cell line[376]. However, this does not explain the development of PD in individuals with non-enzyme depleting *GBA1* polymorphisms, including E326K[375].

Other proposed hypothesis leading to neuro-toxicity in PD patients with *GBA1* mutations include[16;352]:-

1. Disturbed autophagy, a lysosomal dependent process, leading to the reduced catabolism of α-synuclein.
2. Induction of endoplasmic reticulum stress and proteasomal dysfunction leading to the impaired degradation of α-synuclein.

3.1.4 *GBA1* MUTATIONS AND GAMMOPATHY

Despite the association between *GBA1* heterozygotes and PD, it is unclear from the literature, whether carriers for a Gaucher gene mutation have other features of GD including an elevated incidence of paraproteinaemia. Several hypotheses, including chronic immune stimulation, decreased tumour surveillance, the accumulation of lipid-laden macrophages and the elevation of B-cell proliferating cytokines, have all been suggested to play a role in the development of gammopathy in GD[66]. If carrier status for a *GBA1* mutation was found to confer an elevated risk of paraproteinaemia then hypotheses linked to disease burden would become less credible. Moreover if *GBA1* heterozygotes were demonstrated to have an elevated incidence of plasma cell neoplasms then gene linkage studies to exclude the co-inheritance of a cancer gene would require investigation.

Rosenbloom *et al.* (2009) performed *GBA1* mutational analysis on 95 patients with multiple myeloma including 18 Ashkenazi-Jews. In those of Ashkenazi-Jewish descent, 2 had mutations, however, none were identified in those of non-Jewish ethnicity[354]. Their study was of insufficient sample size or power to disprove an association.

Potentially, an associated between carrier status and a monoclonal band could be approached either by screening for *GBA1* mutations in patients with an M-band or by testing the parents of patients with GD i.e. obligate carriers, for a paraprotein. Additionally, siblings of patients with GD, that were found to be carriers of mutations, could be included.

3.1.5 HYPOTHESIS

The following hypotheses are tested in this chapter based on the introduction above:-

Hypothesis 1:- Disease severity predicts for monoclonal and polyclonal gammopathy in GD

Rationale:- Several cohorts of GD patients have been shown to have an increased incidence of gammopathy. Prior literature has been inconclusive and not sufficient to answer whether markers of disease severity including biomarkers predict for immunoglobulin abnormalities.

Methods:- Analysis of clinical data pertaining to local patients with GD at the Royal Free Hospital.

Hypothesis 2:- *GBA1* is a cancer gene or B-cell promoter and carrier status leads to an elevated risk of paraproteinaemia.

Rationale:- Patients with *GBA1* mutations, homozygotic or heterozygotic, have an elevated risk of Parkinson's disease. It is currently unknown whether carrier status confers an increased risk of monoclonal gammopathy.

Methods:- *GBA1* gene analysis of non-GD patients who have an established paraprotein from Jewish descent.

Hypothesis 3:- Patients with myeloma acquire pathology in the monocytic lineage similar to that seen in GD.

Rationale:- Non-GD individuals with haematological malignancies including multiple myeloma have infiltrates of foamy macrophages by light microscopy on bone marrow examination (pseudo-Gaucher cells). It is unclear whether these cells or their precursors

acquire similar biochemical disturbances to monocytic cells derived from patients with GD.

Methods:- Monocyte glucocerebrosidase activity and plasma chitotriosidase activity.

3.2 METHODS

3.2.1 LOCAL GAUCHER DISEASE COHORT – ROYAL FREE HOSPITAL

Clinical information was recorded on 75 patients with type I Gaucher disease who were treated at the Lysosomal Storage Disorders Unit, Royal Free Hospital. Data was recovered from local pathology databases and the patients case notes. Analysis was performed on the same cohort of patients, depending on treatment status, at 2 different time points. Data was recorded on treated patients immediately prior to commencing ERT and from the most recent evaluation as of the 1/2/2009. Approximately 85% of patients had gone on to receive ERT for a minimum of 12 months. For untreated patients, clinical and pathological parameters were recorded as of 1/2/2009.

Data capture included disease severity (Zimran severity score), immunoglobulin levels, serum electrophoresis, splenectomy status, platelet count, baseline glucocerebrosidase activity, *GBA1* genotype, chitotriosidase activity, ferritin, acid phosphatase (ACP), serum-angiotensin converting enzyme (S-ACE) and treatment status.

3.2.2 CHITOTRIOSIDASE ACTIVITY

Plasma was collected from healthy controls and patients with GD, myeloma and MGUS. All samples were processed on the same day as collection and stored at -80°C until analysis. Chitotriosidase activity was determined by fluorometric assay in accordance with the method of Hollak *et al*. 1994 (see section 2.8.4).

3.2.3 GLUCOCEREBROSIDASE ACTIVITY

PBMCs were isolated from peripheral blood and the glucocerebrosidase activity of monocytes was determined by a flow cytometry as described (section 2.8.5). Monocytes were identified based on forward and side scatter characteristics with a minimum of 10,000 gated events being analysed.

3.2.4 *GBA1* MUTATION ANALYSIS

3.2.4.1 STATITICAL CONSIDERATIONS

Prior to commencing screening for *GBA1* mutations, several statistical scenarios were modelled. Statistical analysis was provided by Richard Morris (Department of Primary Care and Population Sciences, Royal Free Hospital). This was performed using an available online application (http://www.swogstat.org).The following assumptions were made in the statistical models presented below:-

(a) Gaucher Gene Carrier frequency in the Ashkenazi-Jewish population – 1/12

(b) Incidence of a paraprotein in the non-GD Jewish population (>50 in age) – 3%

(c) Carriage of a Gaucher gene mutation in the general population 1:800

Option (1):-

How many Ashkenazi-Jews with a paraprotein are required to demonstrate an association with a mutated *GBA1* allele?

(i) Relative risk 2.0 with power of 80% together with $p<0.05$ = 118 people

(ii) Relative risk 1.5 with power of 80% together with $p<0.05$ = 382 people

[1 tailed statistical test, assuming a control population:sample population of 32:1]

Option (2): How many people from a general population, of unspecified ethnicity, are required to demonstrate an association between a paraprotein and a mutated *GBA1* allele?

Assuming a relative risk of 2.0, power of 0.8, $p<0.05$ and carrier frequency of 1/800 in the general population, approximately 9,043 people are required for recruitment.

[1 sided statistical test]

Option (3): How many relatives of patients with GD with a *GBA1* mutation (>50 years in age) are required in order to demonstrate a casual association with a blood paraprotein?

(i) Relative risk 2.0, power 0.8 with a $p<0.05$ = 353 people

(ii) Relative risk 3.0, power 0.8 with a $p<0.05$ = 123 people

(iii) Relative risk 3.0, power 0.9 with a $p<0.05$ = 150 people

[1 tailed statistical test, 99:1 control population:sample population]

Option 2, screening the general population, is unfeasible based on recruitment numbers, expense and manpower. Option 3, is also not plausible, as over 300 individuals are required to demonstrate a relative risk of 2.0. There are approximately 90 patients with GD under the care of the Royal Free Hospital with their relatives geographically distributed across the United Kingdom. Therefore, we opted for option 1, with the aim of recruiting approximately 120 patients.

3.2.4.2 DEMOGRAPHICS OF THE JEWISH POPULATION IN LONDON

Data collected from the 2001 census, for the first time, described the demographics of the Jewish population in London (Piggott and Lewis 2006; Greater London Authority). They reported a total of 149,789 people of Jewish ancestry in London, comprising 2.1% of all individuals, of which 59% were less than 50 years old. London is divided into boroughs with the borough of Barnet containing approximately one third of all London Jews and about 18% of all Jews nationally. Harrow, Brent, Redbridge and Camden are the next four London boroughs with the highest number of Jewish residents (Table 3-1). Hackney and Haringey contain a relatively young Jewish population. Criticisms of the 2001 census include the absence of information detailing Jewish denomination. Ashkenazi Jews have the highest carriage rate of *GBA1* mutations and therefore targeting these populations would require less numbers in order to gain sufficient statistical power. Haringey and Hackney have a comparatively large number of orthodox Jews.

	Jewish Average Age	Jewish Population Number per London borough	Jewish Population Percentage per London borough
Barnet	52	46,686	14.8
Redbridge	46	14,796	6.2
Harrow	47	13,112	6.3
Camden	43	11,153	5.6
Hackney	33	10,732	5.3
Westminster	49	7,732	4.3
Brent	52	6,464	2.5
Haringey	38	5,724	2.6
Enfield	48	5,336	2.0
Kensington and Chelsea	43	3,550	2.2

Table 3-1. Demographics of the Jewish population in the 10 London boroughs with the highest number of Jewish residents (2001 census).

3.2.4.3 RECRUITMENT OF JEWISH PATIENTS WITH A PARAPROTEIN

Approximately 90 patients of Jewish origin with a paraprotein were identified at the Royal Free Hospital. Individuals were identified based on recorded ethnicity and a detectable M-band. However, the majority of these patients had MGUS, and many were receiving yearly review. In order to address recruitment difficulties, amendments were made to the original local ethics and R&D submission. This allowed for multi-site involvement, an invitation to participate by post, telephone communication and phlebotomy in the patients' home. Ethical and R&D approvals were obtained from each participating hospital trust. These trusts were identified as serving London boroughs of high Jewish ethnicity. These included:-

1. Royal Free Hospital (RFH) Hampstead NHS Trust – Camden, Barnet
2. Barnet and Chase Farm Hospitals NHS Trust – Barnet, Enfield
3. Barking Havering and Redbridge NHS Trust – Redbridge
4. North West London Hospitals NHS Trust – Brent, Harrow

Signed informed consent was acquired from all participating patients. An example of a patient information and consent form is found in Appendix 1. All patients who were found to be homozygous or compound heterozygotes for *GBA1* mutations were reviewed at the Lysosomal Storage Disorders centre, Royal Free Hospital. Those found to be carriers of a *GBA1* mutation received counselling by telephone and their General Practitioner was asked to refer for genetic counselling, if appropriate.

3.2.4.4 *GBA1* MUTATION SCREENING – STRATEGY

DNA was isolated from stored whole blood (thawed from -80°C) or immediately from freshly isolated PBMCs. As described by Hruska *et al.* (2008), 84.3% of all *GBA1* mutations, including 13 of the 15 commonest mutations, are contained within exons 5-11 (Hruska *et al.* 2008). Amplification of these exons was performed using two initial PCRs encompassing blocks 5-7 and 8-11. Primers were designed to be specific for the *GBA1* gene and not the *GBAP* pseudogene, as used by Stone *et al.* (2000). Sequence primers were exon specific. In a minority of samples, due to degraded DNA, exon 6 was amplified and sequenced using the same set of exon specific primers. Two common mutations found in the Jewish population, 84GG and IVS2+1, are contained in a 255bp fragment in exon 2. An allele specific PCR was used to detect the 84GG mutation. The IVS2+1 allele was detected by performing an enzyme digest on the amplified 255bp product. Methods are described in detail in section 2.10.

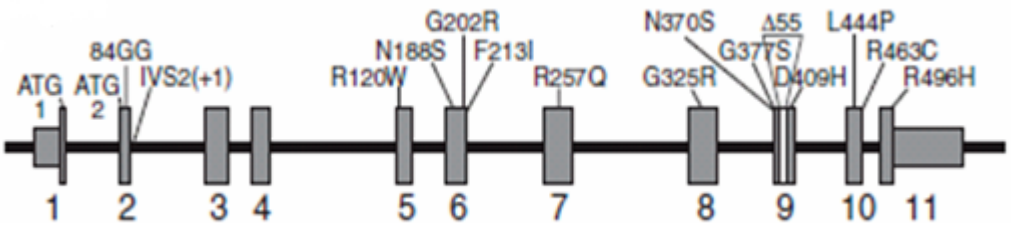


Figure 3-1. G*BA1* gene identifying the location of the 15 most commonly mutated alleles. Adapted from Hruska *et al.* (2008).

3.3 RESULTS

3.3.1 DESCRIPTION OF GAMMOPATHY IN GD PATIENTS

Based on the analysis of 75 patients with GD, 10 (13.3%) were identified as having a blood paraprotein and their demographics are described in Table 3-2 (Patients 1-10). Patient 8 died from sepsis in 2001 just prior to commencing chemotherapy for multiple myeloma with a paraprotein of 56g/l.

As of the 1/2//2009, the average age of those with a paraprotein was 59.6 years (range 27-87; n=9). The mean paraprotein level was 8.4g/l with 9/10 being IgG in isotype and 6/10 being kappa light chain expressing. Patient 5 had bi-clonal low level IgG paraproteins of differing light chain class. Patients 4 and 10 had undergone splenectomy prior to developing a paraprotein. Interestingly, patients 2 and 3 were male siblings of similar age. Patients 2 and 10 had persistently elevated IgA immunoglobulins (polyclonal), despite ERT, whereas all other treated patients with a paraprotein had normal serum levels within their unaffected immunoglobulin classes. All patients had radiological evidence of skeletal disease. Patient 2 developed a paraprotein whilst on ERT but it is unclear whether patient 10 had a paraprotein prior to commencing disease-modifying therapy. All other patients had a documented paraprotein before starting ERT.

Five patients with a paraprotein had undergone bone marrow examination:-

(A) Patient 3. Bone marrow examination (November 2007) revealed an infiltrate of 25% Gaucher cells and approximately 5% plasma cells. His skeletal disease was attributed to GD rather than myeloma-related end organ tissue impairment (ROTI) and he was classified as having MGUS.

(B) Patient 4 underwent bone marrow examination in June 2008 when his paraprotein level was 15g/l. The trephine revealed an infiltrate of 50% Gaucher cells without elevation in plasma cell numbers. Skeletal manifestations were attributed to GD and he was staged as having MGUS.

Patient	Current Age/yrs (1/2/2009)	Paraprot-ein g/l	Working Diagnosis	Ig Class	Mutation	Peak Chito Activity	Chito Mutation	Asplenic	Bone Disease/ BMB Score
1	83	9	MGUS	IgG-λ	IVS2+1 N370s	2265	Not known	No	Yes Moderate
2	50	4	MGUS	IgG-κ	N370s L444P	16503	Wild	Yes	Lumbar – 6 Femora - 5
3	44	21	MGUS	IgG-κ	N370s L444P	9498	Wild	No	Lumbar – 3 Femora - 4
4	54	15	MGUS	IgG-κ	RecNcil ?2nd allele	9450	Not known	No	Lumbar - 5 Femora - 6
5	78	8	ASM	IgG-λ IgG-κ	N370s L444P	12475	Hetero	No	Lumbar - 6 Femora - 6
6	87	4	MGUS	IgA-κ	N370s N370s	5042	Wild	No	Lumbar - 4 Femora -6
7	27	1	MGUS	IgG-λ	N370s ?2nd allele	6644	Not Known	No	Lumbar – 6 Femora – 5
8	Died 2001, aged 58 (Sepsis)	56 (at death)	?MM	IgG-λ	N370s N370s	12290	Not Known	Splenectomy post development of a paraprotein	Yes Bi-lateral hip replacement
9	46	1	MGUS	IgG-λ	N370s L444P	2664	Wild	No	Yes moderate
10	68	4	MGUS	IgG-κ	R463C/?	10714	Wild	Yes	Severe skeletal disease

Table 3-2. Characteristics of 10 Gaucher disease patients with a blood paraprotein. Bone Marrow Burden (BMB) score is calculated on T1 image intensity on MRI, T2 image intensity and the distribution of bone marrow anomaly (maximum score 8). ASM (asymptomatic myeloma), MM (multiple myeloma) and MGUS (monoclonal gammopathy of undetermined significance).

(C) Patient 5 underwent a bone marrow trephine in August 2006 revealing numerous Gaucher cells and an infiltrate of plasma cells, which in part clustered into organised sheets. He was classified as having asymptomatic myeloma, as dictated by International Myeloma Working Group (IMWG) criteria, and the bony disease was reported as being consistent with GD.

(D) Patient 8 had a bone marrow examination in 2001, the same year as his death, revealing only a low-grade plasma cell infiltrate together with the presence of numerous Gaucher cells. Initially he was classified as having asymptomatic myeloma with his skeletal disease attributable to GD. However his paraprotein subsequently peaked at 56g/l. This gentleman had decreased peripheral blood counts and impaired performance status. He was suspected to have multiple myeloma but died of septicaemia prior to commencing chemotherapy.

(E) Patient 10 underwent bone marrow examination in 2004 and 2008 for low blood counts. Gaucher cells were evident but an increase in plasma cell numbers was not reported. His skeletal disease was attributed to GD

3.3.1.1 GLUCOCEREBROSIDASE ACTIVITY – GAMMOPATHY

Initially it was hypothesised that baseline glucocerebrosidase activity would be predictive for the development of gammopathy (polyclonal or monoclonal). Patients with type III disease have more severe disease and generally lower residual enzyme activity. Baseline glucocerebrosidase activity was considered a marker of disease severity for this comparison. This data was available for 32 patients, which included 6 individuals with a paraprotein. GD patients without gammopathy (0.1±0.1; Activity/Mid-reference range; mean±SD) had identical enzyme activity to those with gammopathy (0.1±0.1; p=0.98; Mann Whitney test). Baseline glucocerebrosidase activity was equivalent in those with and without a paraprotein. (p=0.62; Figure 3-2)

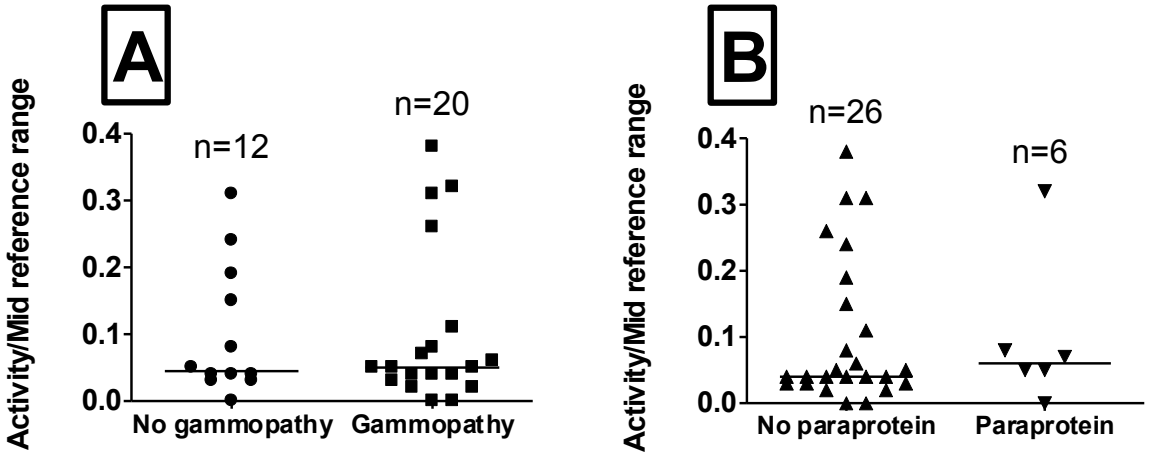


Figure 3-2. Enzyme activity of GD patients/mid-range value of the reference range in those with (A) gammopathy and (b) paraproteinaemia.
Plotted line represents median.

3.3.1.2 INCIDENCE OF POLYCLONAL GAMMOPATHY

The absolute levels of IgG, IgM and IgA were analysed in treatment-naive individuals, excluding immunoglobulins with an associated paraprotein (Figure 3-3). In total, 59% (37/75) had polyclonal gammopathy with IgM being the commonest affected immunoglobulin. Elevated IgM, IgA and IgG levels were seen in 38%, 20% and 22% of patients respectively. Gammopathy affecting two immunoglobulin classes were present in 9/75 individuals with a further 3/75 patients having increases in all 3 isotypes (IgA, IgG and IgM).

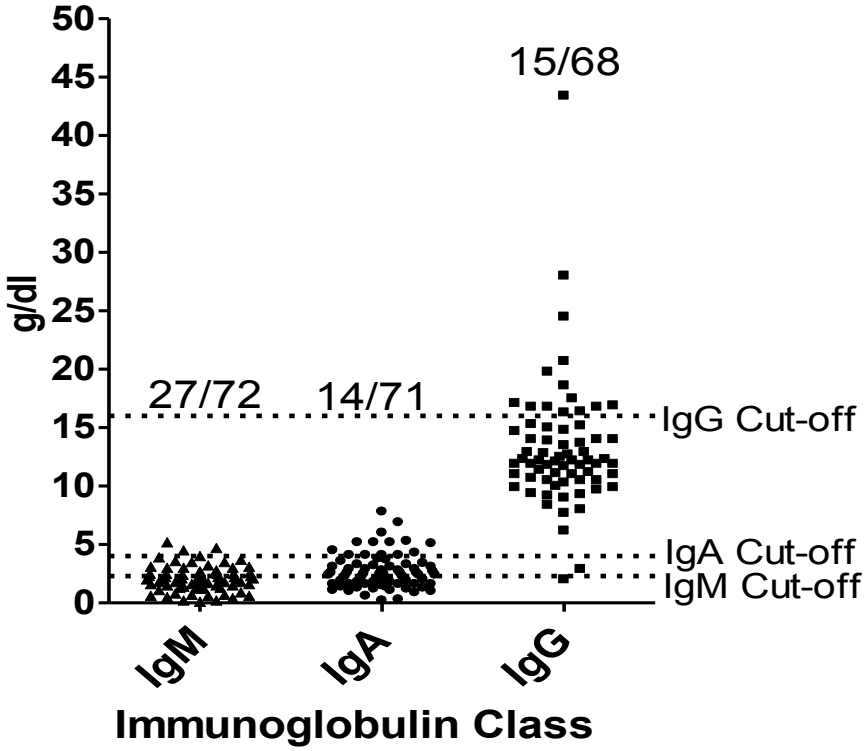


Figure 3-3. Graph representing the absolute levels of immunoglobulins in untreated GD individuals. The number of patients with elevated values is reported above each immunoglobulin isotype. The broken line indicates the upper limit of normal range.

In treated individuals (>12 months), polyclonal increases in IgM, IgA and IgG were seen in 9%, 12.5% and 9.4% respectively. In comparison to ERT-naïve individuals, polyclonal elevation in more than one immunoglobulin class was not seen.

3.3.1.3 POLYCLONAL GAMMOPATHY – DISEASE MODIFYING THERAPY

ERT led to a highly significant reduction in the absolute immunoglobulin levels of IgM (n=55 p<0.0001; paired data; Wilcoxon Signed Rank Test), IgA (n=54; p<0.0001) and IgG (n=51; p<0.0001) isotypes in individuals who had received treatment for a minimum of 12 months (Figure 3-4).

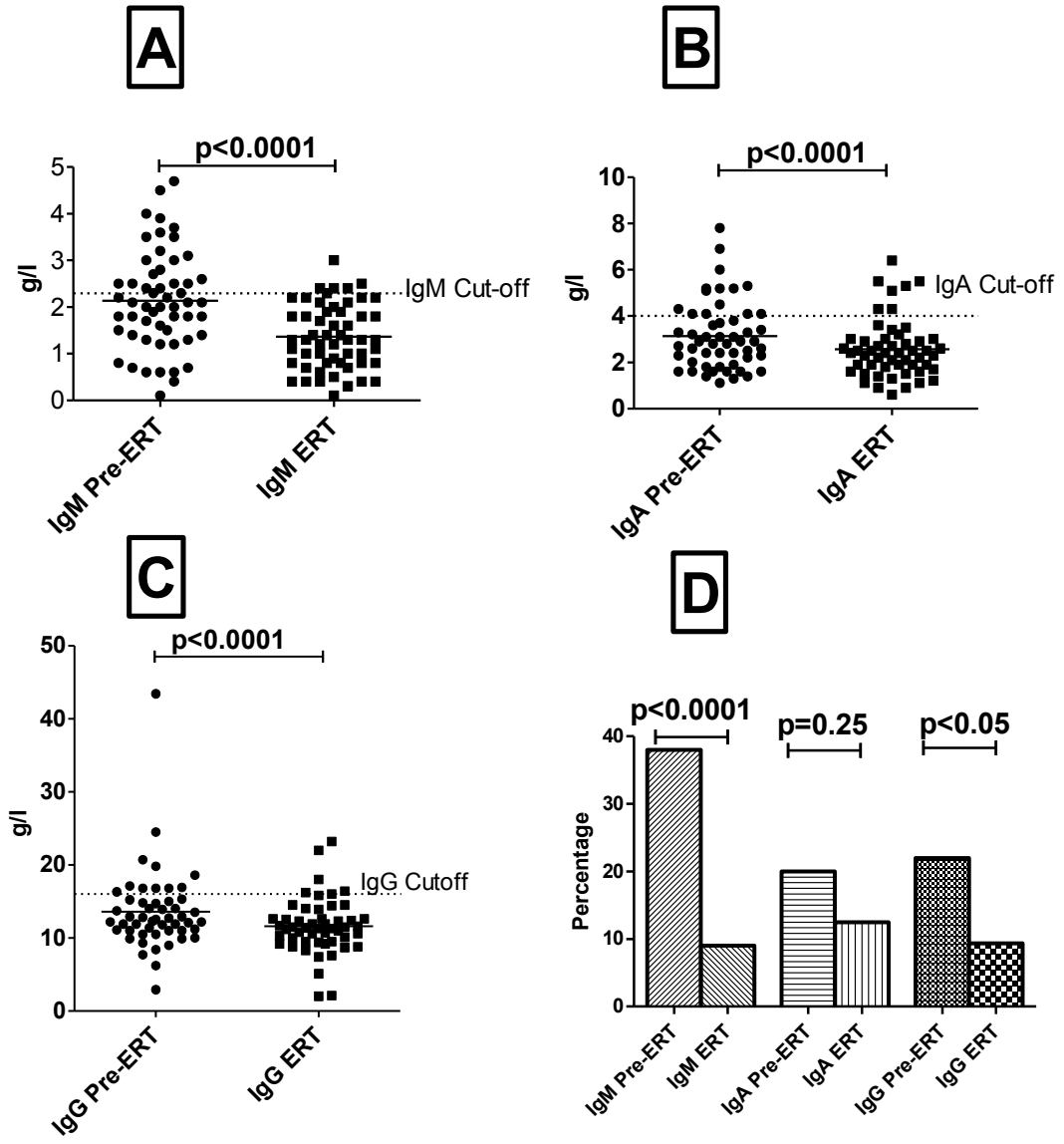


Figure 3-4. Absolute immunoglobulin levels pre and post a minimum of 12 months therapy. (A) IgM, (B) IgA and (C) IgG isotypes (Wilcoxon Signed Rank Test). Horizontal line represents mean, broken line indicates upper limit of normal. (D) Graph represents the percentage of patients with elevated immunoglobulins pre and post introduction of ERT (Fishers Exact Test).

The treated cohort had received therapy for a median of 9 years and the percentage of individuals with elevated IgM or IgG levels reduced with treatment. Therefore, ERT is effective in attenuating polyclonal gammopathy.

3.3.1.4 MONOCLONAL GAMMOPATHY - DISEASE MODIFYING THERAPY

The paraprotein levels in patients on ERT, with quantified paraproteins over several years, are plotted below. Interestingly, the patient with multiple myeloma (patient 8), experienced a large fall in his paraprotein post splenectomy (Figure 3-5). However, this was transient, despite concomitant treatment with ERT. Although the other 6 patients had received ERT for several years, no decrease in the paraprotein was observed.

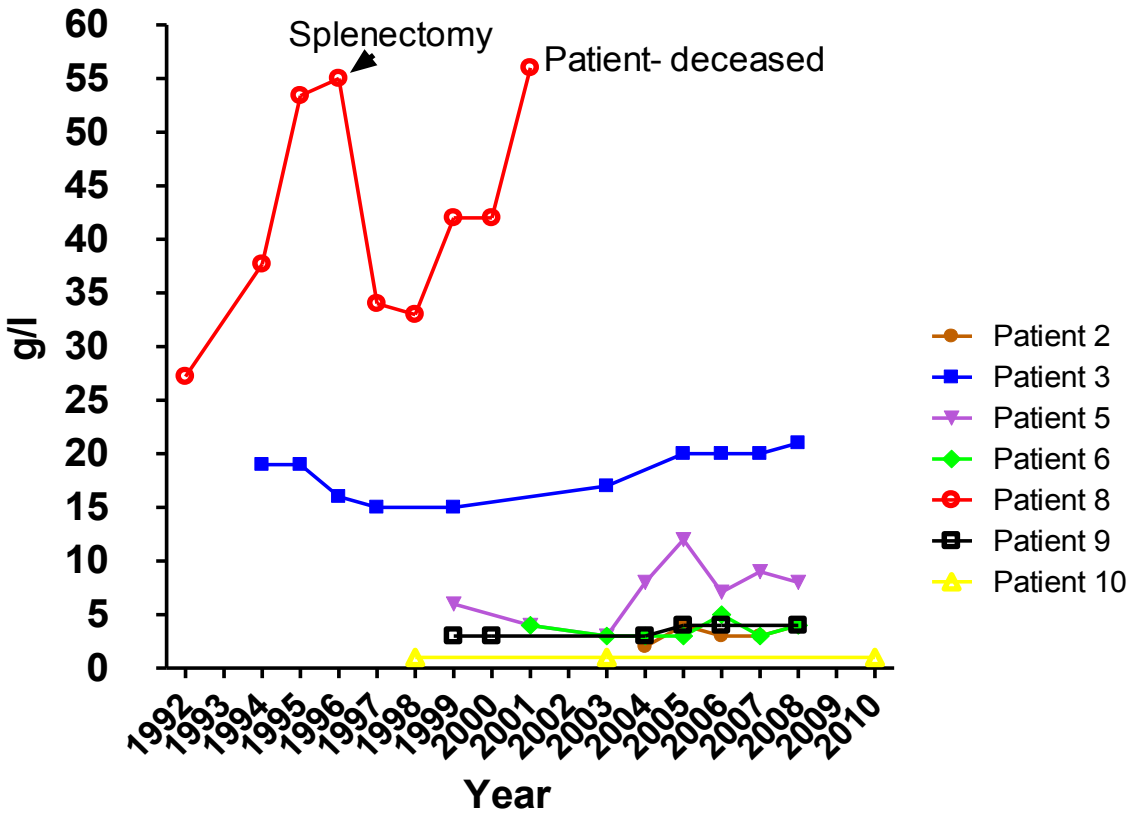


Figure 3-5. Trend of paraprotein over time for GD patients with a longstanding M-band on ERT. ERT was started in 1997, 1995, 2001, 2008, 2000, 1994, 1998 and 1998 in patients 2, 3, 5, 6, 8, 9, and 10 respectively.

3.3.1.5 GAMMOPATHY AND CHITOTRIOSIDASE ACTIVITY (ERT NAÏVE)

Peak chitotriosidase activity is reported below in treatment-naïve individuals and for patients with a paraprotein or polyclonal gammopathy. Patients with gammopathy, monoclonal or polyclonal, had significantly elevated chitotriosidase activity compared to those with normal immunoglobulin analysis ($p<0.05$; Figure 3-6). Patients with a paraprotein had a similar chitotriosidase activity (8134±5743 nmol/hr/ml; n=29; mean±SD) compared to GD individuals with normal immunoglobulins (5436±4120; n=10; p=0.14). These comparisons assume an equal distribution of null chitotriosidase gene mutations between analysed groups. Sub-analysis of patients with functional chitotriosidase genes (wild type), demonstrated higher chitotriosidase enzyme activity in those with gammopathy, monoclonal or polyclonal, compared to those with normal immunoglobulins ($p<0.05$).

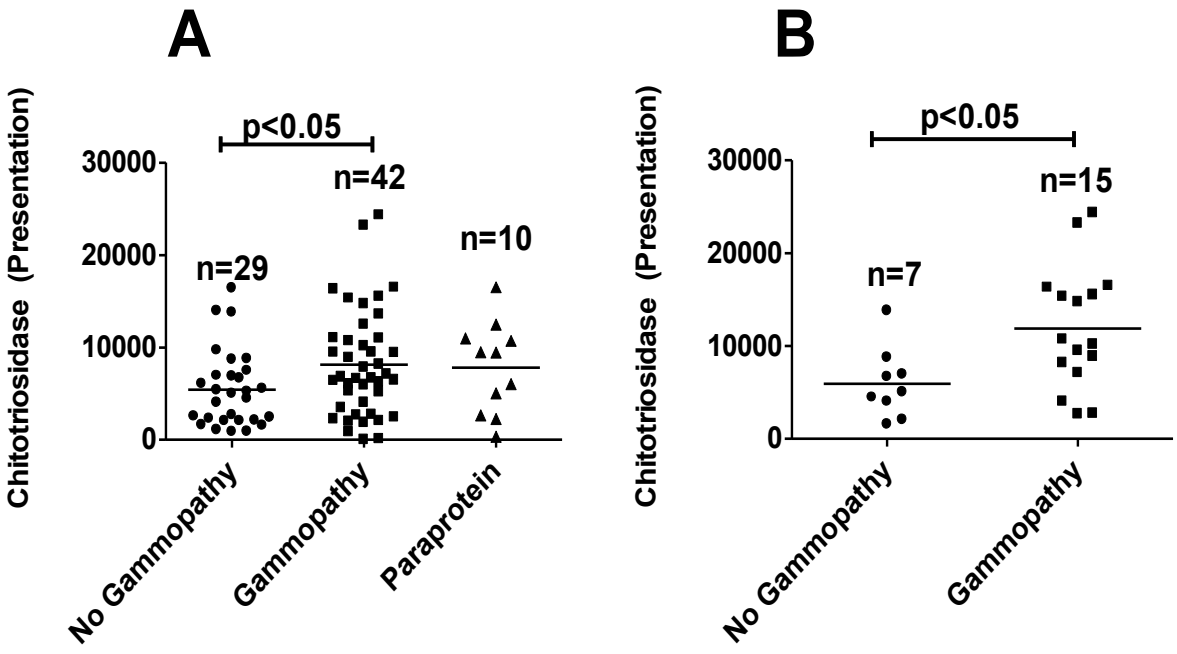


Figure 3-6. Graph showing chitotriosidase activity (nmol/hr/ml) in patients, with and without gammopathy, prior to the instigation of disease modifying therapy.
(A) an unselect GD population or (B) in those known to have functional chitotriosidase genes. Horizontal line indicates mean. Para (paraprotein).

Chitotriosidase activity did not correlate with the absolute levels of IgM (r=-0.02, p=0.86), IgA (r=0.21, p=0.08) or IgG (r=0.33, p<0.01). This comparison holds true when comparing those with functional chitotriosidase genes.

3.3.1.6 GAMMOPATHY AND CHITOTRIOSIDASE ACTIVITY (ERT ERA)

Patients with gammopathy had increased chitotriosidase activity (2759±2665 nmol/ml/hr; mean±SD) compared to those without gammopathy (1288±1796; p<0.01). This observation holds true for those with functional chitotriosidase genes (p<0.01; gammopathy 2881±2139 versus normal immunoglobulins 847±742; Figure 3-7). In patients receiving ERT, chitotriosidase activity was not significantly elevated in individuals without a paraprotein (1503±1957 nmol/ml/hr; n=64) when compared with those with a paraprotein (3460±3347; n=9; p=0.07).

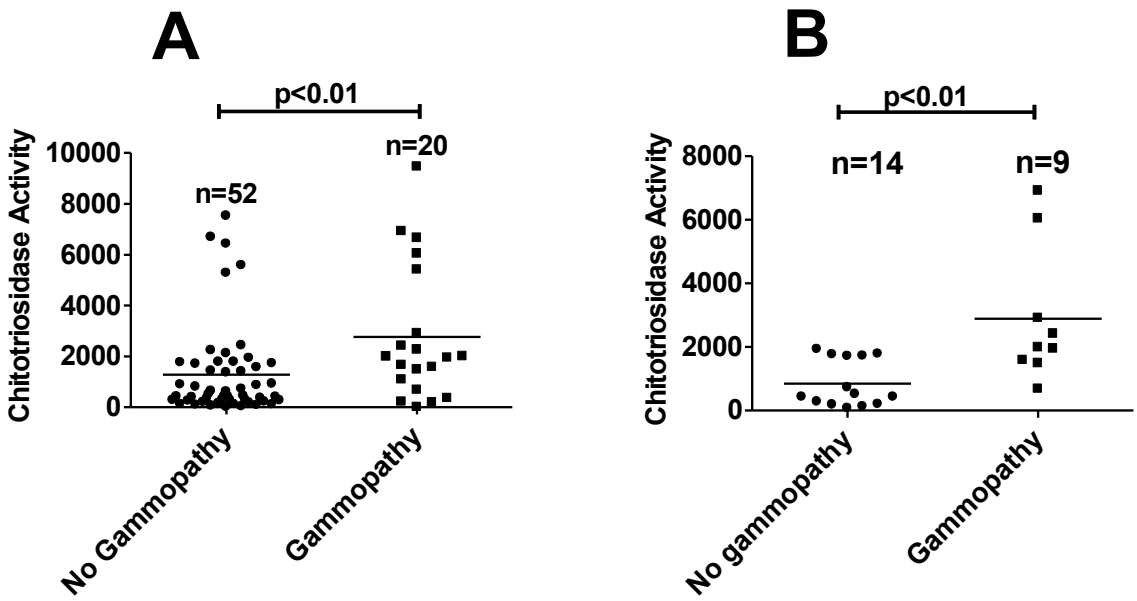


Figure 3-7. Chitotriosidase activity of GD patients with gammopathy or normal immunoglobulins in the ERT era.
(A) Unselect cohort. (B) GD patients with functional chitotriosidase genes. Horizontal line indicates mean.

3.3.1.7 ACID PHOSPHATASE, SERUM ACE AND FERRITIN IN PATIENTS WITH GAMMOPATHY (ERT NAÏVE)

Additional biomarkers, of GD severity (acid phosphatase, ferritin and S-ACE), were analysed in those with normal immunoglobulins, polyclonal gammopathy and individuals with a paraprotein. Available data, in ERT naïve patients, shows serum acid phosphatase and S-ACE to be elevated in those with gammopathy compared to those with normal immunoglobulins. Ferritin levels were not discriminatory between GD patients with and without gammopathy (Table 3-3).

	No gammopathy	**Gammopathy**	**p-value**
Acid Phosphatase (ng/ml) **Normal:- 2.6-6.2**	9.2±3.2 (n=30)	11.6±5.1 (n=41)	<0.05
S-ACE (U/ml) **Normal:- 8-55**	99.9±75.3 (n=31)	143.7±103.7 (n=43)	<0.05
Ferritin (ng/ml) **Normal:- 30-340**	441±372 (n=30)	515±263 (n=43)	0.4

Table 3-3. Serum levels of acid phosphatase, S-ACE and ferritin in GD individuals with gammopathy (monoclonal or polyclonal) or normal immunoglobulins.

Patients with a paraprotein had a higher serum acid phosphatase level than those without ($p<0.05$; Table 3-4) but had equivalent levels of S-ACE and ferritin.

	No Paraprotein	**Paraprotein**	**p-value**
Acid Phosphatase (ng/ml) **Normal:- 2.6-6.2**	10.1±3.6 (n=62)	13.3±8.1 (n=9)	p<0.05
S-ACE (U/ml) **Normal:- 8-55**	117.8±86.4 (n=65)	179.7±136.4 (n=9)	p=0.07
Ferritin (ng/ml) **Normal:- 30-340**	466.9±373.9 (n=65)	591.8±281.8 (n=9)	p=0.15

Table 3-4. Serum levels of acid phosphatase, S-ACE and ferritin in GD individuals with and without a paraprotein.

3.3.1.8 ZIMRAN SEVERITY INDEX – GAMMOPATHY (ERT NAÏVE)

The Zimran severity score (ZSS) was able to be calculated in 56 ERT naïve patients, 10 of who had a paraprotein. Patients with polyclonal gammopathy had an identical ZSS (8.1±3.2; mean±SD) to those without gammopathy (9.6±5.1; p=0.2; Figure 3-8). However, patients with a paraprotein had a ZSS (12.2±5.3) ($p<0.05$) higher than individuals with polyclonal gammopathy (8.9±4.2).

GD patients with a paraprotein did not have a higher ZSS than GD patients (normal immunoglobulins plus polyclonal gammopathy) without a paraprotein (p=0.06).

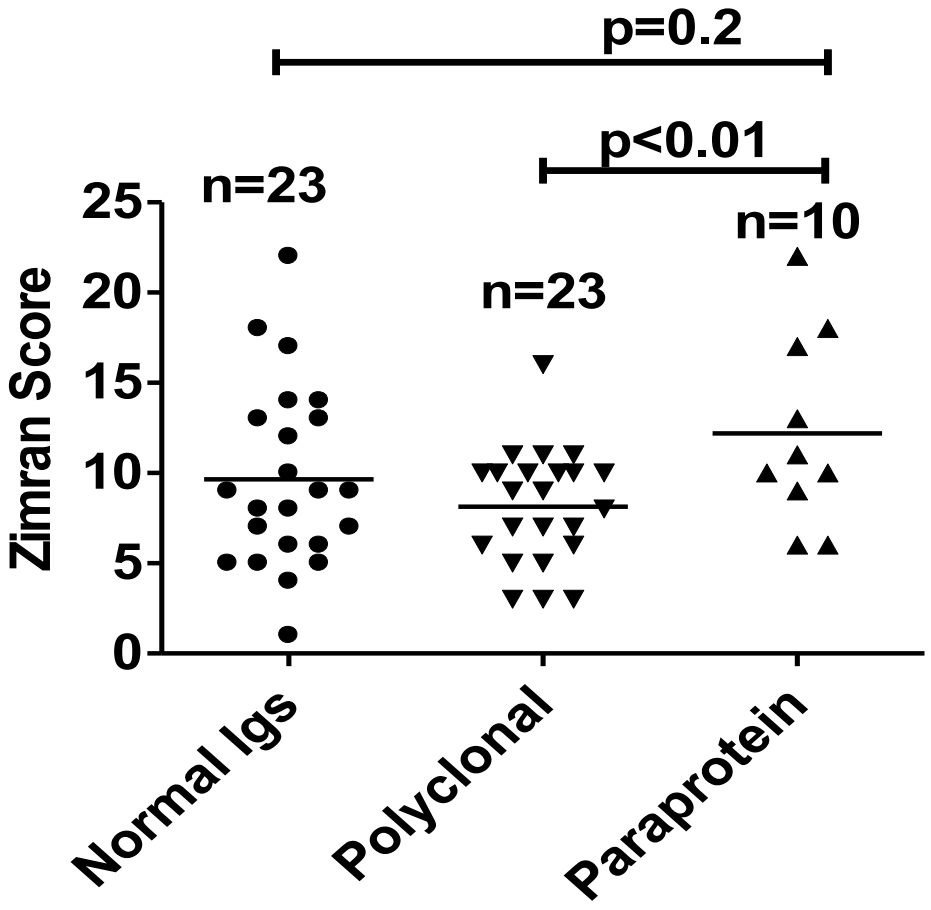


Figure 3-8. Zimran score in patients with normal immunoglobulins, polyclonal gammopathy and those with a paraprotein.

3.3.1.9 PLATELET COUNT – GAMMOPATHY

Thrombocytopenia in individuals with GD is most often due to visceral sequestration, particularly in the spleen. In addition, infiltration of the bone marrow, by lipid-laden macrophages, may compound this. Therefore, a low platelet count may be a surrogate marker for disease severity. Prior to ERT, GD patients with gammopathy had a significantly lower platelet count (102±65x10^9/L; mean±SD) than those with normal immunoglobulins (166±138x10^9/L; p=0.02; Figure 3-9). The platelet count did not differ in patients with or without a paraprotein (p=0.19). However 9/10 individuals with an M-band had a lower platelet count than the mean count seen in those without a paraprotein.

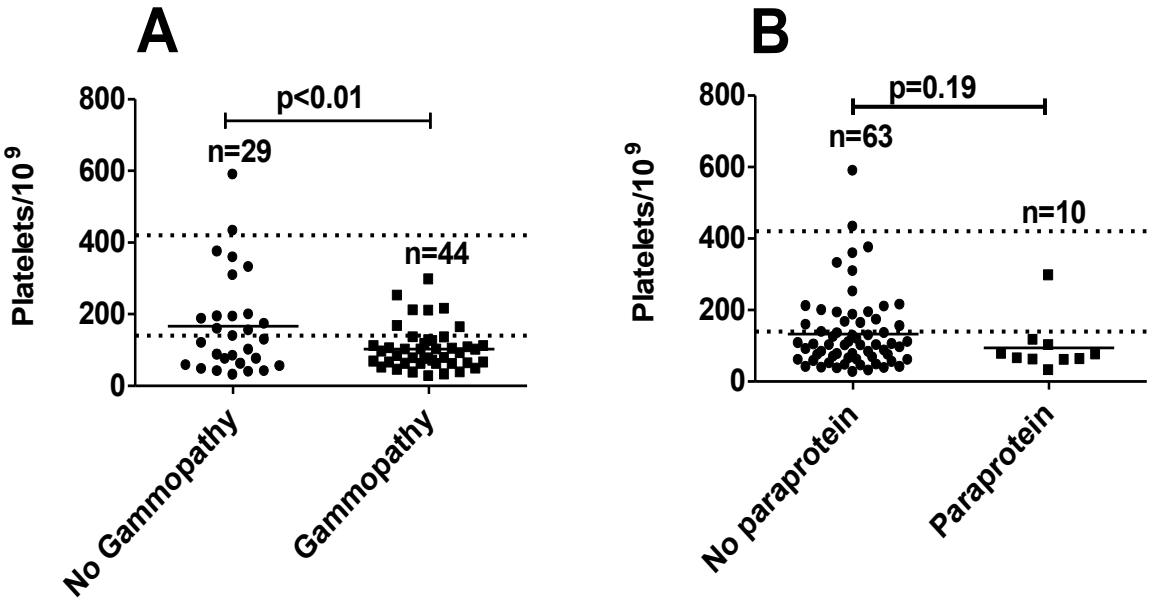


Figure 3-9. Platelet counts in patients with GD that are ERT naïve.
(A) No gammopathy versus gammopathy. (B) No paraprotein versus paraprotein. Horizontal line indicates mean, broken lines signifies normal range.

In those on ERT for a minimum of 12 months, the platelet count was identical in those with gammopathy (210±110x10^9/L; n=35; mean±SD) and those without gammopathy (187±89x10^9/L; p=0.26).

3.3.1.10 SPLENECTOMY

Historically, splenectomy was performed in treatment naïve individuals for refractory cytopenia, symptomatic relief or for diagnostic purposes. Therefore, splenectomy may

be an indicator of disease severity. The incidence of splenectomy was determined in 75 treatment-naïve individuals and was found to be equivalent between GD patients with gammopathy (asplenic 9/44 patients) and with normal immunoglobulins (9/31; p=0.6). In individuals with a paraprotein, only 2/10 patients had undergone splenectomy.

3.3.1.11 *GBA1* MUTATIONS - GAMMOPATHY

GBA1 genotype was available in 72 patients (Table 3-5) of which 22 alleles were unidentified. The most common genotype in those with a paraprotein was N370S/L444P, whereas N370S/N370S was more prevalent in those with normal immunoglobulins. There was no statistical link between any allele or genotype, including N370s homozygosity and the presence of gammopathy (monoclonal or polyclonal).

Genotype	No Gammopathy (n=29)	Gammopathy Monoclonal or polyclonal (n=33)	Paraprotein (n=10)
N370S/N370S	7	7	2
N370S/L444P	5	6	4
N370S/?	6	10	1
L444P/?	2	1	0
L444P/R463C	3	0	0
N370S/D409H	1	3	0
N370S/84GG	1	4	0
RecNcil/?	0	0	1
R463C/?	0	0	1
N370S/C1263del55	1	0	0
N370S/IVS2+1	1	0	1
N370S/V349L	1	0	0
R463C/R463C	0	0	0
N370S/RecNcil	0	1	0
N370S/R120Q	1	1	0

Table 3-5. *GBA1* genotype in individuals with normal immunoglobulins, polyclonal gammopathy or normal immunoglobulin analysis.

3.3.2 CHITOTRIOSIDASE ACTIVITY – PLASMA CELL DISORDERS

Chitotriosidase activity was determined in healthy controls, patients with GD and non-Jewish individuals with either MGUS or multiple myeloma. In addition, chitotriosidase enzyme activity was determined in Jewish patients with a paraprotein, including those with MGUS and malignant plasma cell neoplasms.

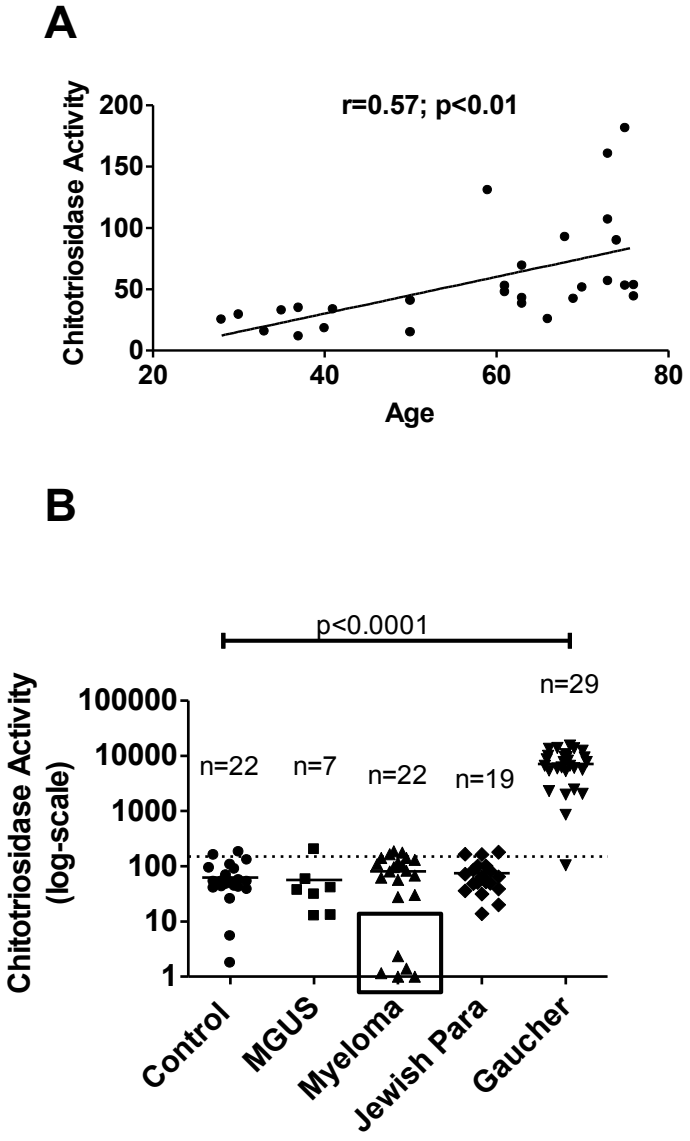


Figure 3-10. Chitotriosidase activity in non-GD patients with gammopathy and GD patients. (A) Chitotriosidase activity (nmol/ml/h) plotted against age in samples derived from healthy controls. (B) Chitotriosidase activity (log scale) in individual groups. Boxed population indicates a small cohort of outliers within the group derived from non-Jewish myeloma patients. Dashed line indicates the upper limit of normal range. Group means indicated by horizontal line.

Chitotriosidase activity has been reported to increase with age[89] and higher enzyme activities were recorded in healthy controls of advancing age (Figure 3-10). Therefore, all analysed groups were aged-matched to within a decade. The mean age and chitotriosidase activity in the analysed groups are reported in Table 3-6.

Group	Mean age	Chitotriosidase Activity Mean±SD
Healthy Controls	67.7	63.0±47.4
MGUS (Non-Jewish)	70.6	56.5±66.4
Myeloma (Non-Jewish)	64.8	81.3±61.1
Paraprotein (Jewish)	72.6	74.9±48.9
Gaucher Disease	48.1	2244±2076

Table 3-6. Mean age and chitotriosidase activity (nmol/ml/hr plasma) within individual analysed groups.

Patients with GD had a significantly higher enzyme activity than healthy controls ($p<0.0001$). Chitotriosidase activity was identical in healthy controls, Jewish patients with a paraprotein and non-Jewish patients with either multiple myeloma or MGUS.

Boxed individuals in the non-Jewish-derived myeloma group (n=5, with low or near absent chitotriosidase activity), may represent a population with null chitotriosidase genes. In addition, these patients were all on immunomodulatory drugs (thalidomide or lenalidomide), as either maintenance or treatment. Disease burden within the boxed group, varied significantly, from frank relapse to plateau post-autograft. These patients had all received at least 1 prior course of chemotherapy. However, there were many patients, with a similar disease stage or treatment profile, that were part of the myeloma cohort, non-boxed, with unremarkable chitotriosidase activity.

3.3.3 MONOCYTE GLUCOCEREBROSIDASE ACTIVITY

A flow cytometric assay was used to determined glucocerebrosidase activity in monocytes derived from healthy controls (n=5), patients with GD (n=5) or non-Jewish patients with myeloma (n=5). In addition, the monocytes of three Jewish individuals

with myeloma were analysed. Patients with GD had low enzyme activity compared to healthy controls (p=0.002). However, glucocerebrosidase activity was not lower in individuals with myeloma irrespective of ethnicity.

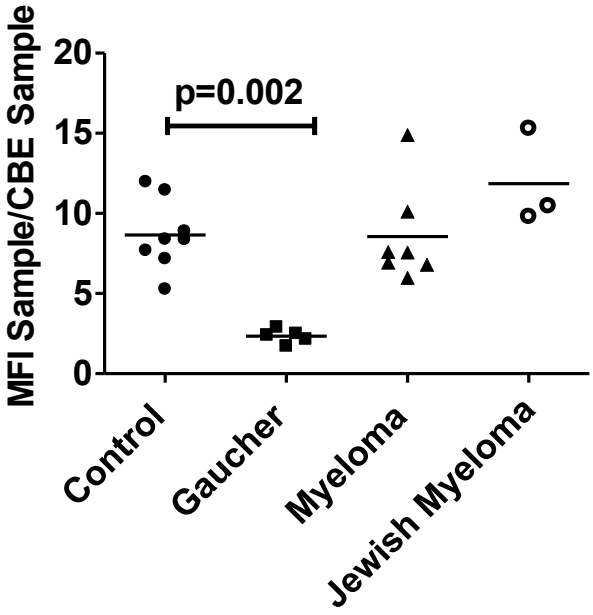


Figure 3-11. Monocyte Glucocerebrosidase activity in controls, non-GD individuals with myeloma and patients with GD.
Monocyte glucocerebrosidase activity as determined by the MFI of liberated fluorochrome from the carrier substrate di-β-glucopyranoside. Enzyme activity reported as a ratio to a paired sample pre-treated with CBE. Horizontal line indicates mean.

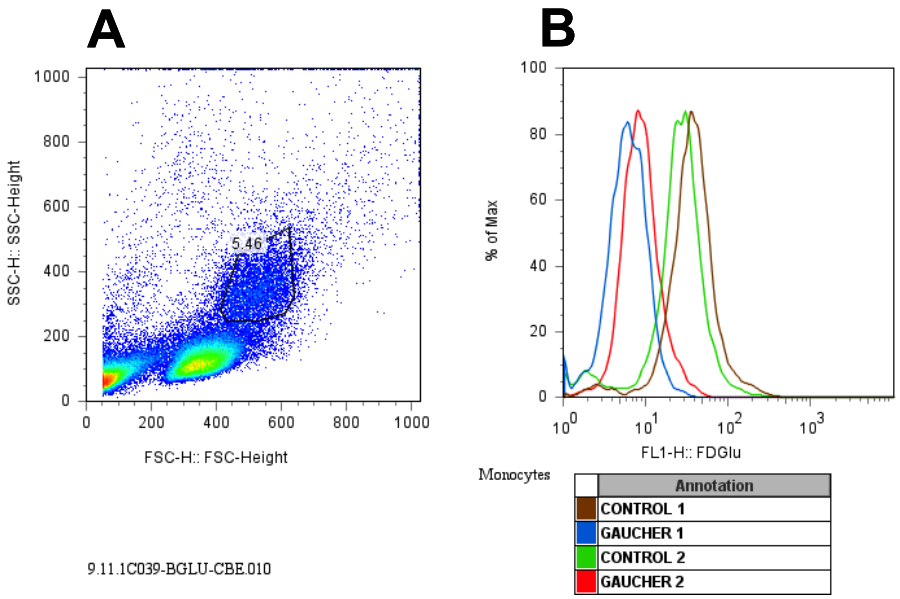


Figure 3-12. (A) Monocytes were identified based on forward and side scatter characteristics. (B) Representative histogram showing a reduction in liberated fluorescein from samples derived from GD patients compared to control.

3.3.4 *GBA1* SCREENING

Initially, a small cohort of DNA samples was processed from healthy controls and patients with either multiple myeloma or MGUS, in order to assess the suitability of screening for *GBA1* mutations using a manufactured strip-test (ViennaLabs, Vienna, Austria). The kit provides material for the isolation and *in vitro* amplification of DNA sequences for the commonest *GBA1* mutations and recombinant alleles by reverse hybridisation. Amplified products are biotinylated and hybridised to a test strip with immobilised oligonucleotide sequences (wild and control sequences). Results were interpreted using a streptavidin-alkaline phosphatase reporter system. As expected, the strip-test did not identify any healthy controls of non-Jewish ethnicity, as having mutated *GBA1* alleles. However, two individuals of Jewish descent were newly identified as having *GBA1* mutations. Patient 1 had multiple myeloma and was found to be a compound heterozygote (N370S/R496H). Patient 2 was a heterozygote for N370S and had a longstanding low-level paraprotein (MGUS). Based on these initial findings a combination of allele specific PCR and DNA sequencing was employed to maximise the detection frequency of *GBA1* mutations.

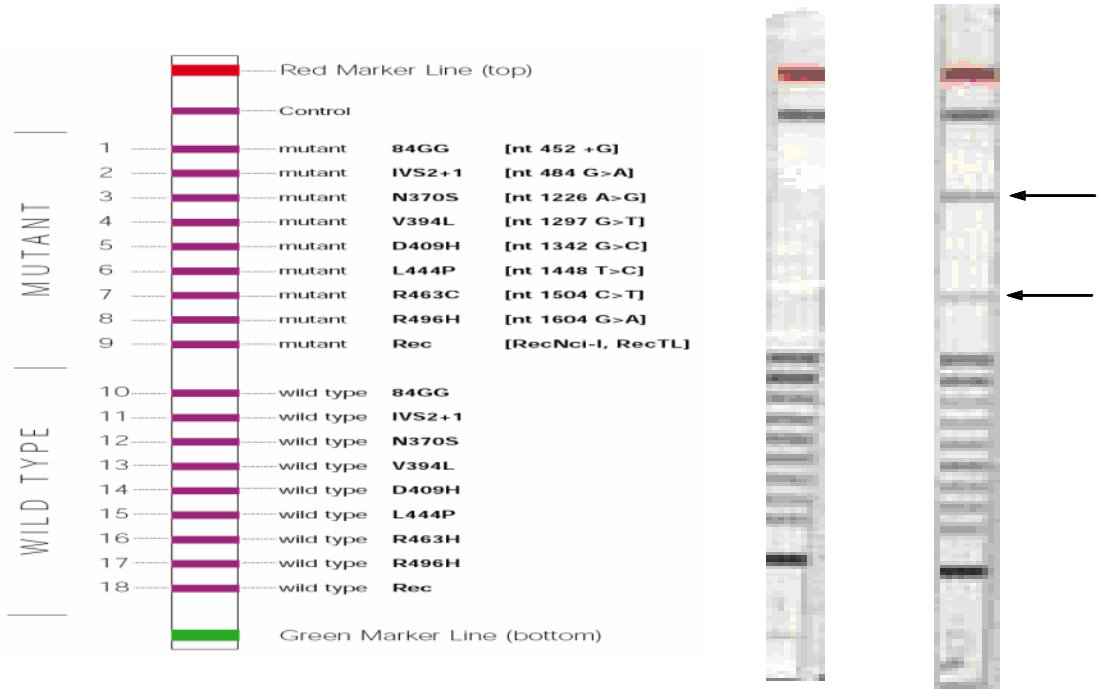


Figure 3-13. *GBA1* analysis. Schematic diagram, normal strip test and compound heterozygote N370S/R496H (from left to right).

	Number	Jewish	Unrecorded Ethnicity	Positive Results
Myeloma	25	3	5	1 (Jewish)
MGUS	1	1	0	1 (Jewish)
Healthy Controls	6	0	0	0

Table 3-7. Results of G*BA1* mutation screening using a pre-manufactured strip-test.

3.3.4.1 *GBA1* SCREENING STUDY - DEMOGRAPHICS

In total, 77 patients of Jewish ethnicity were recruited from 4 NHS hospital trusts. 48/65, 7/65 and 10/65 of individuals reported had Ashkenazi, Sephardic or mixed/ unknown Jewish ancestry (data was not collected on 12 individuals). Paraproteinaemia was most commonly associated with MGUS or myeloma. A minority of patients had lymphoplasmacytic lymphoma (LPL), AL-amyloidosis, or chronic lymphocytic leukaemia (CLL); Table 3-8.

	RFH	NWLH	BCF	BHR
Patients Identified from Database Search	96	18	30	19
Successful *GBA1* analysis/Total Patients recruited	49/60	6/8	14/17	8/9
Male:Female	21:28	3:3	7:7	4:4
MGUS (n=)	20	4	9	7
Myeloma (n=)	16	1	4	1
Smouldering Myeloma (n=)	3	0	0	0
Amyloidosis (n=)	2	0	0	0
Low grade Lymphoma e.g. LPL/CLL	1CLL 7 LPL	1 Follicular Lymphoma	1 LPL	0

Table 3-8. Patient demographics of identified Jewish patients with a paraprotein at 4 London based NHS Trusts.
RFH (Royal Free Hospital NHS Trust Hampstead), NWLH (North West London Hospitals NHS Trust), BCF (Barnet and Chase Farm Hospitals NHS Trust) and BHR (Baking Havering Redbridge NHS Trust). LPL (lymphoplasmacytic lymphoma), CLL (Chronic lymphocytic leukaemia).

3.3.4.2 *GBA1* MUTATION SCREENING – RESULTS

Of the 77 patients, 7 were found to be heterozygous for a *GBA1* mutation. In addition, one patient was found to be a compound heterozygote (N370S/R496H). All mutations were confirmed based on two sequences. Approximately 10.4% of individuals (1:9.6) were found to have an abnormal *GBA1* locus with a mutated allele frequency of 5.8% (9/154). N370S was the commonest allele in the 8 individuals with an abnormal *GBA1* gene.

	cDNA	Exon	Protein	Mutation	Disease	Status
Patient A	c.1226A>G	9	p.Asn409Ser	N370S/Wild	MGUS	Carrier
Patient B	c.1226A>G	9	p.Asn409Ser	N370S/Wild	LPL	Carrier
Patient C	Allele Specific PCR	2	-	84GG/Wild	Myeloma	Carrier
Patient D	c.1226A>G c.1604G>A	9 11	p.Asn409Ser p.Arg535His	N370S/R496H	Myeloma	Gaucher disease
Patient E	c.1093G>A	8	p.Glu365Lys	E326K/Wild	AL Amyloidosis	Carrier
Patient F	c.1226A>G	9	p.Asn409Ser	N370S/Wild	Myeloma	Carrier
Patient G	c.1226A>G	9	p.Asn409Ser	N370S/Wild	MGUS	Carrier
Patient H	c.1226A>G	9	p.Asn409Ser	N370S/Wild	MGUS	Carrier

Table 3-9. Description of Jewish patients with a paraprotein harbouring *GBA1* mutations.

Compared to a reported 1:12 carrier rate in Ashkenazi Jews, an increased frequency of mutated *GBA1* alleles was not observed in those with a paraprotein (Z test=0.67, normal distribution, p=0.5). Statistical analysis using an online calculator (http://www.swogstat.org), revealed the power of this study, based on the pre-study statistical criteria specified to be 57.2% (alpha error=0.05, RR=2, control population:sample population 32:1; one tailed test). In a two-tailed test, power decreased to 41% (Statistics performed by Richard Morris in the Department of Primary Care and Population Sciences, Royal Free Hospital).

Patient C, a myeloma patient, was found to be a carrier for 84GG, a single base insertion, in exon 2. This is demonstrated on gel electrophoresis following allele-specific PCR (Figure 3-14).

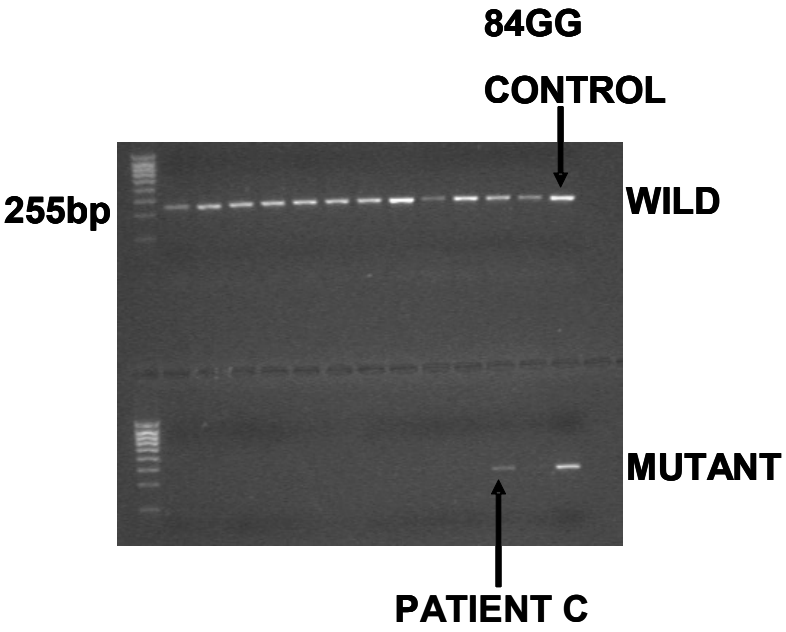


Figure 3-14. Gel electrophoresis (2% agarose) demonstrating patient C to be heterozygous for the 84GG single nucleotide insertion.
The last lane contains a known patient with GD who is a heterozygote for 84GG.

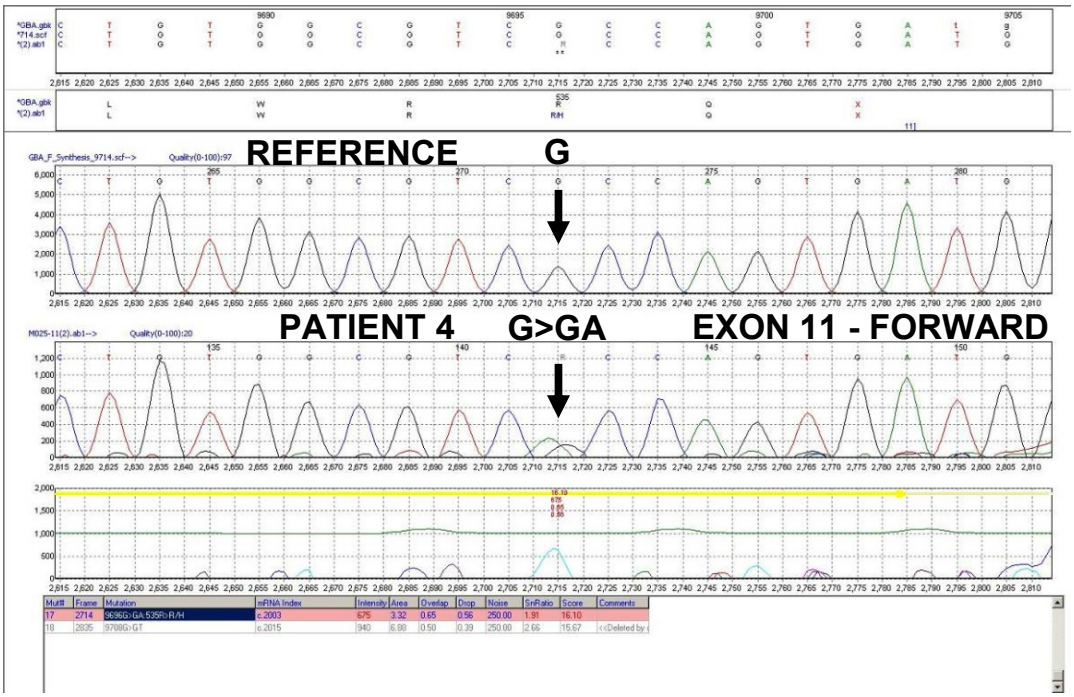
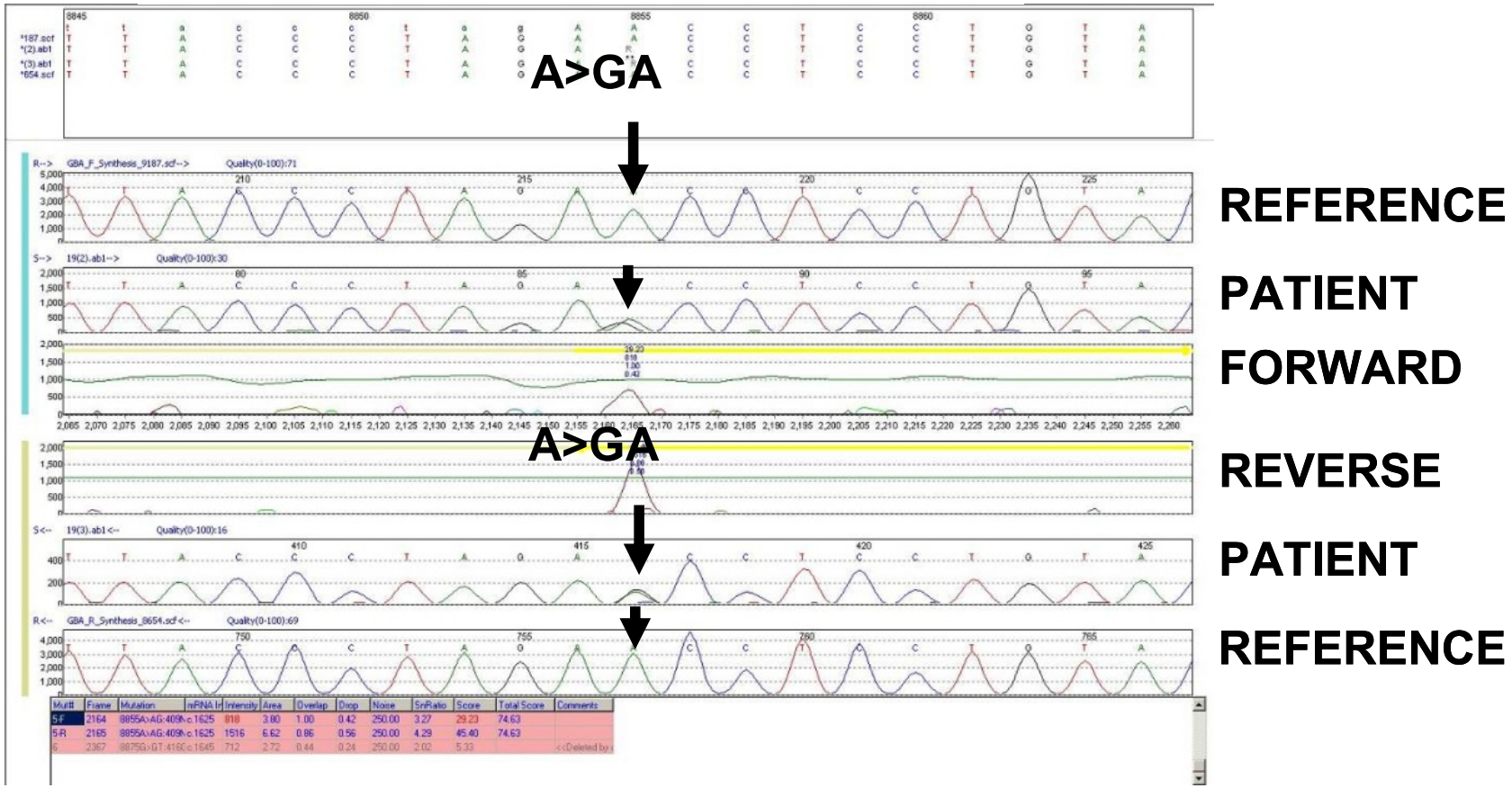


Figure 3-15. Patient D. The illustrated mutation reflects heterozygotic expression of the exon 11 mutation R496H (G >GA).

Figure 3.16. Patient H. The illustrated mutation reflects heterozygotic expression of N370S (A>GA).



3.3.4.3 DESCRIPTION OF JEWISH PATIENTS WITH *GBA1* MUTATION

A brief description of the patients with *GBA1* mutations is provided below:-

(A) **Patient A (N370S/Wild).** This 79 year old lady originally presented in 2006 with an IgG paraprotein of 21g/l. Based on staging investigations MGUS was diagnosed with 3% plasma cells on a bone marrow specimen. Currently her paraprotein remains stable. Co-morbidity includes idiopathic Parkinson's disease.

(B) **Patient B (N370S/Wild)**. This 63 year old man was diagnosed with lymphoplasmacytic lymphoma in 2006 following investigation for a persistent lymphocytosis. Serum electrophoresis revealed two IgGκ paraproteins of 2g/l and 11g/l. Radiological staging revealed no lymphadenopathy or hepato-splenomegaly. Currently he remains stable and untreated.

(C) **Patient C (N370S/Wild)**. This 81 year old woman was diagnosed with MGUS in 2007 with two low level paraproteins (2g/l and 4g/l) and 6-8% plasma cells on a bone marrow trephine. Currently she is well.

(D) **Patient D (N370S/R496H)**. This 72 years old woman was initially diagnosed with asymptomatic myeloma in 2004 with an IgGκ paraprotein of 25g/l. In 2006 her paraprotein progressively rose and this was associated with a 20% infiltrate of plasma cells on a bone marrow trephine. She had a partial response (PR) to cyclophosphamide, dexamethasone and thalidomide. In 2008 bortezomib and dexamethasone were prescribed for progressive disease, leading to a good PR. She has just commenced lenalidomide/dexamethasone for relapsed disease. Despite being found to be a compound heterozygote for GD, no Gaucher cells were noted on prior bone marrow examination.

(E) **Patient E (E326K/Wild).** This 80 year old lady was diagnosed with multiple myeloma (20-30% infiltrate of plasma cells) complicated by amyloid affecting the oral

cavity, including lips. No paraprotein was present but she had a lambda light chain litre of 507mg/l. Echocardiography and serum amyloid A protein (SAP) scintigraphy demonstrated the absence of further deposition. Currently she remains well with stable disease after completing a course of melphalan, steroid and thalidomide.

(F) **Patient F (N370S/Wild).** This 70 year old gentleman presented in 2008 with IgD myeloma complicated by a thoracic plasmacytoma. Staging investigations revealed a 10% plasma cell infiltrate on bone marrow trephine, skeletal disease and a paraprotein of 3g/l. He was treated with 6 cycles of cyclophosphamide, dexamethasone and thalidomide and received 20Gy radiotherapy to the plasmacytoma. .

(G) **Patient G (N370S/Wild).** This 91 year old gentleman with MGUS has had a stable low level IgG paraprotein <10g/l for the past 7 years.

(H) **Patient H (N370S/Wild).** This 84 year old gentleman has had a stable IgMκ paraprotein since 2008. MGUS was diagnosed post staging investigations including bone marrow examination.

3.4 DISCUSSION

The results presented in this chapter demonstrate a high incidence of monoclonal and polyclonal gammopathy in the local cohort of patients with GD. Data presented here, for the first time, supports the hypothesis that disease severity is a risk factor for gammopathy. ERT naïve patients with gammopathy had higher chitotriosidase activity, serum acid phosphatase (ACP) and serum-ACE together with lower platelet counts than those without gammopathy. In addition, serum acid phosphatase ($p<0.05$) and S-ACE ($p=0.07$) seemed higher in GD patients with a paraprotein compared to those without. Furthermore GD patients with a paraprotein had a higher ZSS than those with polyclonal gammopathy. ERT led to a reduction in all immunoglobulins (IgG, IgM and IgA) and stabilised the level of paraproteinaemia. Macrophage precursors (peripheral blood monocytes), derived from non-GD patients with a paraprotein, had normal glucocerebrosidase activity. Furthermore, non-GD patients with an establish paraprotein, had normal plasma chitotriosidase activity. This data, does not support the hypothesis that non-GD patients with an M-band acquire similar abnormalities within their monocytic-macrophage lineage to that seen in GD. Despite recruiting from 4 NHS trusts serving large numbers of Jewish patients, the number of Jewish patients identified with a paraprotein was low. 8/77 Jewish patients with a paraprotein were found to have *GBA1* mutations (7/77 heterozygotes; 1/77 homozygote). The study was of insufficient power (57.2%) to reject the null hypothesis.

In the RFH cohort, in ERT naïve individuals, 59% had polyclonal gammopathy, in keeping with the frequency reported by other investigators[74;139;162]. Furthermore, 12/75 (16%) individuals had elevation in two or more immunoglobulin classes (IgM, IgA or IgG). Wine *et al.* (2007) reported polyclonal gammopathy in 21/23 (91%) untreated paediatric patients with type I GD, of which 11/21 children (52%) had increased levels of more than one immunoglobulin[234]. The higher incidence of polyclonal gammopathy reported in this small cohort is likely to reflect a group of patients with severe disease as the mean age at presentation was 3.9 years. These findings support the chapter hypothesis that gammopathy is more prevalent in those with severe disease. In the RFH cohort many individuals presented in adulthood or remain untreated. IgM was the commonest elevated immunoglobulin (38%) in

keeping with the report by Khalifa *et al.* 2010[235] describing gammopathy within a juvenile population of GD patients. The majority of published cohorts in GD patients have reported IgG to be the predominantly elevated isotype[43;74;138;139]. However, a small increase in the reference range for IgM, would have led to many more individuals within the RFH cohort to having normal levels.

A few comments should be made regarding the staging of monoclonal gammopathies in GD. Firstly, it is difficult to distinguish between myelomatous bone disease and pathology secondary to their storage disorder as compression fractures, lytic disease and osteoporosis are common to both[377]. Therefore, GD patients with progressive skeletal pain, with biochemical evidence of a clonal disorder (Bence Jones proteinuria, elevated serum light chains or paraproteinaemia), should be considered for a CT guided bone biopsy to exclude bony plasmacytoma, given the high reported incidence of plasma cell neoplasms. In addition, anaemia, a feature of symptomatic myeloma, is also a hallmark of GD. Based on agreed international diagnostic criteria (International Myeloma Working Group; IMWG) clonal plasma cell populations greater than 10% on bone marrow examination cannot be classified as MGUS[378]. The percentage of plasma cells on bone marrow sampling may be underestimated in GD due to dilution by accumulated Gaucher cells. This may lead to the incorrect staging/classification of the plasma cell disorder. Non-GD patients with asymptomatic myeloma (plasma cell infiltrate >10%) have a higher rate of progression to symptomatic myeloma than those with MGUS (plasma cell infiltrate<10%)[203;227] and therefore require more frequent follow-up and appropriate counselling.

Data from a Minnesota based population study of more than 20,000 participants reported paraproteinaemia in 3.2%, 5.3% and 7.5% of individuals older than 50, 70 and 85 years in age respectively[201]. From the cohort of 75 RFH patients with GD, 13.3% (n=10) were found to have a blood paraprotein. As of 1/2/2009, the mean age of GD patients with a paraprotein was 59.6 years (n=9; excluding patient 8, deceased). The incidence of monoclonal gammopathy varies with race and is more common in blacks than caucasians (Aged>70, 8.4% versus 3.8%; $p<0.001$)[204]. Data is lacking regarding the incidence of paraproteinaemia in Ashkenazi Jews without GD.

The high incidence of paraproteinaemia in the RFH cohort of GD patients was in keeping with prior reports[74;81;138;139].However, Brautbar *et al.* (2004), in an Israeli based study of GD patients, reported a low incidence of paraproteinaemia (1%)[43]. Speculatively, the number of patients with GD identified as having a clonal plasma cell disorder, in their study, may have been increased by screening for a urinary M-band or an abnormal serum light chain ratio. Whether GD patients have a higher incidence of light chain or non-secretary myeloma is unknown. In a cohort of 13 GD patients (German/Dutch) with monoclonal gammopathy only one individual was reported to have light chain myeloma[81], in keeping with the low number of individual case reports[136]. An abnormal free light chain ratio is a strong predictor for progression from MGUS to multiple myeloma, in non-GD patients[379]. Interestingly, de Fost *et al*. (2008) reported an abnormal free light chain ratio in all three of their GD patients that had or subsequently progressed to malignant disease[81]. In contrast, only 1/9 of their patients with MGUS had an abnormal kappa/lambda ratio.

M-band size, IgA isotype, the percentage of bone marrow plasma cells and an abnormal free light chain ratio are risk factors predictive of progression to multiple myeloma from MGUS[380]. An American based study showed that approximately 1% of MGUS patients per year develop multiple myeloma[203]. It is unknown at present whether these risk factors or if specific cytogenetic abnormalities are able to predict for transformation to malignant disease in those with GD. Data capture should be encouraged via collaborative registries to detail disease characteristics and the natural history of those with plasma cell disorders.

Kappa light chain expressing paraproteins are more common than lambda in non-GD populations[200;201]. In the RFH GD cohort there was a small predominance of kappa light chain containing paraproteins (6κ:5λ). Nine out of the ten Gaucher patients, reported above, had IgG paraproteins, which is the most frequent isotype in non-GD patients with either MGUS or multiple myeloma[201]. No patient with GD had an IgM paraprotein, although, this observation maybe limited due to the low number of individuals with a monoclonal band (n=10). An IgM paraprotein, in two population studies in non-GD patients, was reported to be the monotypic immunoglobulin in 17-23.7% of individuals[200;201]. Combining the RFH patients with published cohorts of

GD patients with paraproteinaemia, an IgM band was found in 4/31 (12.9%) individuals[74;81;138;139]. Patients with IgM MGUS are more likely to progress to Non-Hodgkin Lymphoma (NHL), chronic lymphocytic leukaemia (CLL), lymphoplasmacytic lymphoma or AL-amyloidosis, than myeloma[211;380]. Speculatively, if GD patients are more prone to non-IgM plasma cell clones, then the probability of progressing to multiple myeloma is higher. Multiple myeloma, in most cohorts, has been reported to be increased in GD[78;129;130]. However, whether GD leads to an increased incidence of NHL is debatable[65;129;381].

It was hypothesised that severity markers, including the ZSS, were predictive for gammopathy in GD. In support of this, ERT naïve patients with an M-band had a higher ZSS (12.2±5.3; mean±SD) than those with polyclonal gammopathy (8.9±4.2). In contrast, others have failed to demonstrate a higher ZSS in GD patients with monoclonal gammopathy[81]. However the ZSS was identical in GD patients with polyclonal gammopathy and normal immunoglobulins. Criticisms of the Zimran score include its insensitivity to reflect disease reduction with disease modifying therapy. The Zimran score was published in 1989 and was intended to predict the clinical severity of *GBA1* genotypes in the pre-ERT era[104]. Newer severity indexes, incorporating scoring domains for biomarkers and the incorporation of modern imaging modalities, may prove more sensitive in identifying individuals at risk of gammopathy (e.g. GauSSI-I score)[106], including malignant disease.

In an Israeli based study, measures of disease severity did not predict for the development of gammopathy[43], although blood biomarkers were not included in their analysis. Based on the data reported above, biomarkers including chitotriosidase activity, ACP, S-ACE and platelet count were more deranged in ERT naïve individuals with gammopathy than those with normal immunoglobulins. Furthermore, chitotriosidase activity remained predictive for gammopathy (monoclonal plus polyclonal) in individuals that had received ERT for a median of 9 years. These data, for the first time, suggest that gammopathy is more common in severely affected individuals, and are in support of the chapter hypotheses. In contrast, untreated individuals with a paraprotein (n=10), perhaps due to low patient numbers, did not have significantly higher chitotriosidase activity (mean±SD; paraprotein 8134±5743; normal immunoglobulins 5436±4120; p=0.14) than those

with normal immunoglobulins. S-ACE, chitotriosidase and acid phosphatase are secreted by macrophages in GD, secondary to lysosomal dysfunction[24]. The ability of GD derived macrophages/osteoclasts to support plasma cell growth and expansion is explored in chapter 4.

Pratt *et al*. (1968) suggested that disease bulk (organomegaly) predicted for polyclonal gammopathy[138]. Although, their cohort of GD patients with hepato-splenomegaly had, in addition, evidence of either aseptic necrosis or chronic infection. However, both infection and inflammation are known causes of polyclonal gammopathy, casting doubt on this conclusion[328]. Macrophages are known to be more prominent in the bone marrow of non-GD patients with multiple myeloma and have been demonstrated to support plasma cell survival in culture[245]. Bone marrow rather than extra-medullary macrophage burden, due to plasma cell co-localisation, maybe more important aetiologically. In keeping with this line of argument, in an American cohort, N370S homozygosity was associated with a high incidence of both skeletal disease and multiple myeloma[52]. In the RFH cohort, infiltrates of macrophages, of upto 50%, have been reported in the bone marrow of GD patients with paraproteinaemia.

Serum ACP was found to be higher in the RFH cohort with gammopathy compared to individuals with normal immunoglobulins and in those with a paraprotein compared to patients without. Tartrate resistant acid phosphatase (TRAP), a specific acid phosphatase, is secreted by osteoclasts derived from the monocytic lineage and is a biomarker of disease severity in GD[86]. GD patients have clinical evidence of osteoclastic over-activity[242] and osteoclasts have been shown to support plasma cell growth *in vitro*[252]. Osteoclasts are more prominent in the bone marrow of non-GD patients with myeloma[262]. It is currently unknown whether elevated serum TRAP is predictive for gammopathy in GD. Hypotheses pertaining to the superior ability of GD macrophages to undergo osteoclastic differentiation and to support plasma cell survival, are explored in chapter 4, using co-culture models.

Ferritin, another biomarker of disease severity in GD[24], was equivalent in those with and without gammopathy (monoclonal or polyclonal). Serum ferritin levels however are modified by other variables, including prior transfusion, gender, concomitant

inflammation, iron deficiency, infection and chronic disorders, such as rheumatological disease and renal failure[382].

Paired data, reported above, demonstrated a reduction in the absolute levels of IgM, IgA and IgG following a minimum of 12 months ERT (median treatment period, 9 years), supporting the hypothesis that disease burden drives gammopathy. In addition, the number of individuals with elevated IgM or IgG levels decreased with ERT. These findings are in keeping with the results of others who reported a reduction in the incidence or level of gammopathy with ERT[43;234;235]. Macrophage derived biomarkers (S-ACE, ACP and chitotriosidase activity) were shown here to be elevated in treatment naïve individuals with gammopathy. IL-6 and IL-10, plasma cell supporting cytokines, are secreted by activated macrophages[24]. A study, reported by de Fost *et al*. (2008), demonstrated that the majority of GD patients with a monoclonal band had elevated IL-10 but normal IL-6 levels[81]. In contrast, other investigators have demonstrated higher plasma IL-6 levels in GD patients with monoclonal gammopathy[74]. Enzyme replacement has been shown, in a study by Allen *et al*. (2007), to reduce IL-10 levels, but not the plasma concentration of IL-6[74].

If polyclonal gammopathy pre-disposes to a plasma cell clone, due to chronic stimulation, then the development of a monoclonal band should be less common in the ERT-era. In the RFH cohort, only patient 2 developed a monoclonal band whilst receiving ERT. In a Dutch cohort, no patient with GD developed a paraprotein on treatment[81]. Accept for patient 8, in the RFH cohort, who already had a large monoclonal band >30g/l, the level of paraproteinaemia remained stable on ERT. In the face of an aggressive plasma cell clone, the introduction of enzyme replacement may be insufficient to prevent progression. Malignant disease developed in 2/12 Dutch patients whilst on ERT, although they were on treatment for only a short period of time[81]. The level of paraproteinaemia, in an Israeli based study, did not regress with ERT[43], in keeping with our findings. This discussion raises important issues, including financial, regarding paraproteinaemia being an indication for ERT in GD.

It has been hypothesised that chronic inflammation/immune stimulation leads to the development of polyclonal gammopathy in patients with GD[162]. Several other inflammatory disorders including malignancy (haematological and non-haematological), liver disease, chronic infection and rheumatological disease have been associated with a persistent elevation in serum immunoglobulins[328]. In fact, in the study performed by Dispenzieri *et al.* (2001), liver disease was the most frequent cause of polyclonal gammopathy. Due to insufficient clinical data, liver volume (or spleen volume) was not correlated with the presence of gammopathy in the RFH cohort. Furthermore, it is unclear if chronic immune stimulation and polyclonal expansion pre-disposes to clonal plasma cell expansion. In a cohort of non-GD patients with polyclonal gammopathy, no individual developed a paraprotein (n=148) after a median follow-up of approximately 6½ years[328]. Data capture was, however, incomplete as 37% of participants were lost to follow-up.

Rheumatoid arthritis (RA), a chronic inflammatory disorder, characterised by joint destruction and enhanced autoimmunity, has been suggested to confer an elevated risk of multiple myeloma in a retrospective study[383]. Bi-clonal paraproteins have been reported in 9.5% (n=21)[329]and 23% (n=13)[81] of patients with rheumatoid arthritis and type I GD, respectively. In comparison, in the general population, bi-clonal bands have been reported to occur in about 3% of individuals with a paraprotein[201]. In the RFH cohort of GD patients, only one individual had bi-clonal gammopathy (10%; 1/10). In two patients with an established IgG paraprotein, a polyclonal increase in IgA was also present prior to the introduction of ERT. The fact that patients with GD and RA are prone to gammopathy, monoclonal or polyclonal, in more than one immunoglobulin class, supports hypotheses relating to the emergence of plasma cell clones secondary to polyclonal stimulation.

It was hypothesised that residual glucocerebrosidase activity may be able to predict which individuals develop gammopathy. Baseline glucocerebrosidase activity, in the RFH cohort of GD patients, did not correlate with the development of gammopathy (monoclonal or polyclonal). However, residual enzyme activity and genotype are not reliable predictors of clinical severity in GD[28;384]. In neuronopathic patients with GD (L444P/L444P), clinical phenotype varied considerably, in addition to fibroblast glucocerebrosidase activity (1-13.3% of control)[56]. Many individuals with

homozygous N370S disease remain clinically silent, in contrast to others who present with rampant disease[52]. Overall N370S/L444P is considered a more severe genotype than N370S/N370S. Despite this, in one small study, residual enzyme activity was found to be lower in those with N370S/N370S (n=4) than individuals with N370S/L444P (n=4)[385]. Furthermore, glucocerebrosidase activity may not predict for storage load as other variables may have a modifying affect. For example, natural variations in the activity of catabolic pathways involved in the breakdown of either globoside or GM1-ganglioside[24] would reduce glucosylceramide accumulation. In addition, decreased ceramide glucosyltransferase activity or increased glucosylceramide deacylase activity would alleviate glucosylceramide deposition.

Specific genotypes did not predict for the development of gammopathy, monoclonal or polyclonal, in the RFH cohort. N370S homozygosity is the commonest genotype reported in the Gaucher registry[28]. However, there were more individuals with N370S/L444P than N370S/N370S in the paraprotein group, reported above. The converse was true in those without monoclonal bands with N370S/N370S being more common. This observation is limited by the fact that two of the patients with a paraprotein were brothers with the same genotype (N370S/L444P). The co-inheritance of other cancer genes, pre-disposing to gammopathy, in these siblings, could not be excluded.

Splenectomy, secondary to traumatic aetiology, in non-GD individuals, does not confer an elevated risk of malignancy[386]. The incidence of splenectomy within a combined German/Dutch cohort of GD patients, was found to be equivalent between those with cancer (n=14), including haematological, and those without (n=117)[78]. Prior splenectomy was not a risk factor for the development of gammopathy, monoclonal or polyclonal, in the RFH cohort. In keeping with the report of de Fost *et al.* (2008)[387], the number of asplenic individuals was similar in those with and without a blood paraprotein. In an early report from the pre-ERT era, splenectomy was suggested to be less common in those with gammopathy (splenectomy rate; 4/6 individuals with normal immunoglobulins, 1/10 with gammopathy)[138]. Compared to other *GBA1* genotypes, multiple myeloma has been shown to be increased in those with homozygous N370S disease[52]. Interestingly in this report, 88.3% of individuals with N370S/N370S had an intact spleen, compared to 61.5% of those with other

genotypes ($p<0.0001$). Skeletal disease rather than visceral bulk, has been reported to be the predominant clinical phenotype in individuals with homozygous N370S disease[52]. These findings add credibility to hypotheses regarding abnormalities within the bone marrow microenvironment being critical to the development of plasma cell neoplasms in GD.

It has been reported that splenectomy leads to decreased IgM levels in patients with type I GD[162] . In fact, in patient 8, reported here, splenectomy was associated with an impressive decrease in the level of paraproteinaemia, 55g/l to 34g/l. Splenomegaly is a common clinical finding in patients with GD[28]. The spleen is a major lymphoid organ and exposes circulating blood cells continuously to processed antigen, foreign or self, by antigen presenting cells in organised follicles. In GD, the spleen is infiltrated by both Gaucher cells and inflammatory immune cells[24]. Accumulated glucosylceramide has been reported to contribute to less than 2% of the excess weight in splenectomised organs from those with GD[24]. It is currently unclear whether deposition of extra-cellular sphingolipid is responsible for the development of a polyclonal plasmacytosis in GD. However, glucosylceramide deposition, in a L444P mouse model, was not demonstrated, despite the development of gammopathy[330]. Shoenfeld *et al.* (1982) showed that lymphocytes from patients with GD, using a migration inhibition factor test, were able to be stimulated by either glucosylceramide or by splenic homogenates from those with GD[162]. They hypothesised that chronic immune stimulation was responsible for the elevated incidence of gammopathy in GD. Glucosylceramide has been shown to stimulate macrophages in culture[388]. This is in keeping with the findings reported in this chapter correlating disease severity to the presence of gammopathy.

In addition, splenectomy leads to the removal of a mass of stimulated immune cells, conferring an immediate reduction in antigen presenting cells and the capacity to produce pro-inflammatory cytokines. B-cell proliferating cytokines, such as IL-6 and IL-10, have been reported inconsistently by investigators to be elevated in the blood of those with GD[74;81]. Gaucher cells, however, have been suggested to manufacture little IL-6 or IL-10[58]. Plasma IL-6 and IL-10 levels have been reported to be identical in asplenic GD patients and those with an intact spleen[74], although in this study 11/22 individuals were receiving ERT. Plasma cells are rarely seen in the peripheral blood,

but may occur in the presence of immune stimulation, infection or plasma cells neoplasms[59]. Alternatively, in patient 8, it could be hypothesised, that clonal plasma cells, due to splenic sequestration, were de-bulked at splenectomy. MacDonald *et al.* (1975)[389] reported the case of a 48 year old GD patient with bi-clonal gammopathy (IgG and IgA) and low blood counts secondary to hypersplenism. A fortnight post splenectomy, her IgA band had reduced by 50% but the IgG paraprotein remained stable. Further analysis revealed a large population of IgA-restricted plasma cells in the spleen, with a minor infiltrate of IgG-restricted plasma cells. The converse was found on bone marrow examination, with IgG-restricted plasma cells pre-dominating. The focal development of a plasma cell clone in the spleen does not necessarily support the concept of chronic antigenic stimulation, as extra-medullary haematopoiesis is known to occur in GD[390].

It was hypothesised that patients with Multiple Myeloma and GD may share similar disturbances in cellular biology, including macrophage pathology. As described above here, individuals with GD have infiltrates of foamy macrophages on bone marrow examination. Previously, an elevated number of bone marrow macrophages have been reported in non-GD patients with myeloma[245]. Boven *et al.* (2004), based on splenic histocytochemistry, suggested Gaucher cells to have the cytokine profile of alternatively activated macrophages. They demonstrated that Gaucher cells have weak expression of both pro-inflammatory and B-cell proliferating cytokines, including IL-6 and IL-10[58]. It could be argued, however, that Gaucher cells, despite weak expression of B-cell proliferating factors, due to their accumulated mass, lead to a net increase in the secretion of these cytokines. This is unclear based on conflicting data from several studies measuring plasma levels[81], but focal increases within the bone marrow micro-environment cannot be excluded and have not be studied in GD. It is unknown from the literature whether Gaucher cells within the bone marrow have a similar Th2 expressing cytokine profile to those in the spleen. As described above, individuals with GD and gammopathy have a higher mean chitotriosidase activity than those with normal immunoglobulin analysis. This finding is suggestive of macrophage bulk being a risk factor for the development of gammopathy. Alternatively, it could be argued, that elevated chitotriosidase activity, reflecting disease severity[83], correlates with increased glucosylceramide deposition, inflammation or other immune defects, capable of facilitating plasma cell expansion.

Based on plasma chitotriosidase activity, non GD-patients, with plasma cell disorders, including multiple myeloma and MGUS, do not share the same spectrum of macrophage pathology as those with GD. In fact non-GD patients with multiple myeloma have similar plasma activity to an aged-matched population. As reported above, compared to healthy controls, macrophages accumulate in the bone marrow of non-GD patients with myeloma and may represent up to 10% of all cells[245]. Data reported above, however, did not control for null chitotriosidase genes. Assuming that all non-GD patients were heterozygotic for a null mutation, chitotriosidase activity would have only been marginally raised above the normal range for elderly individuals[89]. Chitotriosidase activity was found to increase with age, in keeping with the findings of other investigators[89]. Approximately one third of individuals are heterozygotic for a null chitotriosidase mutation with an additional 6% of the general population being homozygous for inactivating mutations[357]. A further criticism of the results reported here is that plasma chitotriosidase activity was not determined at baseline in all non-GD patients with multiple myeloma and therefore may not represent peak enzyme activity. Myeloma chemotherapy may be toxic to macrophages and could be postulated to have lead to a reduction in macrophage activity *in vivo* of treated patients. In addition, due to the decreased turnover of dead cells as chemotherapy progresses, the macrophages of treated individuals may have less apoptotic cells to digest and less saturated glucocerebrosidase activity. However, chitotriosidase activity was not elevated in individuals at presentation, relapse or with refractory disease in this cohort.

Gaucher cells, based on electron microscopic examination, have been shown to have a different ultra-structure to the foamy macrophages present in the bone marrow of patients with haematological cancer (Pseudo-Gaucher cells)[358]. Monocytes, macrophage precursors, based on flow cytometric assay, had equivalent glucocerebrosidase activity in healthy controls compared to non-GD patients with plasma cell dyscrasias (malignant or benign). Thus, non-GD patients with plasma cell disorders do not acquire a secondary deficiency in glucocerebrosidase activity. However, these results do not exclude the development of secondary enzyme deficiency in tissue macrophages that have a longer life span[391]. In-situ hybridisation of bone marrow trephines for glucocerebrosidase mRNA would provide further

information to investigate this hypothesis. Tissue macrophages have significant phagocytic activity and have been shown to polarise towards a phenotype associated with alternatively activated macrophages by glioma cancer stem cells[392]. Therefore, peripheral monocytes or macrophages, extrinsic to the primary tumour, may not develop the same biochemical abnormalities or cytokine profile as those abutting malignant growths.

Jewish patients without GD but with a plasma cell disorder, compared to healthy controls or non-Jewish individuals with MGUS or multiple myeloma, had identical plasma chitotriosidase activity and monocyte glucocerebrosidase activity. Therefore, being of Jewish ancestry seems not to be a risk factor *per se* for developing biochemical abnormalities in the monocytic lineage consistent with GD. Jewish patients included in these results were confirmed to be negative for *GBA1* mutations. Due to the founder effect, there is an increased incidence of specific mutations in *BRCA1/BRCA2*[148], conferring an elevated risk of breast and gynaecological cancer, in Ashkenazi Jews. In addition, certain mutations in *MSH2* and *APC*[393], imparting an elevated risk of colorectal cancer, are reported in the Ashkenazi Jewish population. It is unclear, at present, whether *GBA1* is an additional cancer gene within this population. In addition, although individuals with GD have an elevated incidence of myeloma[130;287], it is unknown whether these individuals co-inherit additional cancer genes conferring an elevated risk of multiple myeloma. Speculatively, homozygosity for *GBA1* mutations alone may be insufficient to confer an elevated risk of malignancy, including clonal plasma cell disorders, in those with GD. *GBA1* is located to the long arm of chromosome 1 and abnormalities of chromosome 1q are not uncommon in individuals with multiple myeloma[394]. One study demonstrated a gain of chromosomal material to 1q in approximately 50% of patients with multiple myeloma, raising speculation of a tumour promoter gene[394]. It could be hypothesised that due to gene linkage the *GBA1* locus is co-inherited with additional tumour promoter genes or oncogenes that confers the observed elevated risk of malignancy in GD. If *GBA1* heterozygosity was associated with an elevated risk of monoclonal gammopathy, then this hypothesis would warrant further investigation.

Although, the ViennaLabs® strip test is an economical and time efficient method for *GBA1* mutation screening, there have been reports within the literature of false

positive results with Gaucher diagnostic kits utilising impregnated oligonucleotide probes[395]. In addition, there are more than 300 reported mutations within the *GBA1* gene and this kit detects only 10[27]. Using this strip-test, based on 635 individuals with non-neuronopathic disease from the International Collaborative Group Gaucher Registry, approximately 9% of mutant alleles would go undetected[33]. Moreover, in the first 4 Jewish patients screened for *GBA1* mutations using the striptest, one heterozygote and one compound heterozygote was identified. For these reasons, it was justifiable to employ an alternative screening strategy. In order to increase the detection of *GBA1* mutant alleles, a combination of DNA sequencing (exons 5-11) and allele specific PCR (IVS2+1 plus 84GG, exon2) were employed. Despite recruiting from 4 NHS trusts serving large populations of Jewish patients, only 163 individuals were identified from electronic hospital databases based on recorded demographics (religion/ethnicity). In total, 92 patients gave informed consent of which 77 had successful *GBA1* analysis. The concurrent treatment of a subset of individuals with chemotherapy may have contributed to the inability to isolate DNA of sufficient quality for analysis. The number of expected patients with a paraprotein, per London borough, assuming a 3% incidence of MGUS in those >50 years, is shown in Table 3-10. Census data in 2001 reported 41% of the Jewish population in London to be >50 years in age (Piggott and Lewis 2006).

London Borough	**Jewish Number (2001 Census)**	**Number >50 years in age**	**Expected number with a paraprotein (>50 years)**
Camden	11,153	4,572	137
Redbridge	14,796	6,066	182
Harrow	13,112	5,376	161
Barnet	16,686	6,841	205
Brent	6,464	2,650	80
Enfield	5,336	2,188	66

Table 3-10. Expected number of Jewish patients >50 years with a paraprotein per London Borough.

The four participating NHS trusts, in this study, would have served the majority of Jewish patients in the London Boroughs listed in Table 3-10. In total 831 Jewish individuals with a paraprotein, aged >50 years, are estimated to be present within these boroughs. In total, assuming these parameters, we identified 20% (161/831) of

those with a monoclonal band. This may not be surprising as the majority of the population with an M-band are well and speculatively remain clinically silent. In addition, ethnicity and religion are often poorly recorded in both patient notes and electronic demographic databases. Individuals with a paraprotein, in the hospital setting, are likely to be skewed towards those with malignant disease or are identified inadvertently post investigation of an alternative co-morbidity.

The fact that only 161 individuals were identified may reflect that *GBA1* heterozygotes are not at an elevated risk of monoclonal gammopathy. Mutated *GBA1* alleles were not increased in 77 Jewish individuals with a paraprotein. However, this study had insufficient statistical power, as described above in section 3.3.4.2, to reject the null hypothesis. In comparison, an association of *GBA1* carrier status and idiopathic Parkinson's disease, has required cohorts of at least 300 people to demonstrate an association[396;397]. Using the observed *GBA1* mutation frequency reported here (8/77), compared to a 1:12 carrier rate, would require the screening of 1347 Jewish patients (http://www.swogstat.org) with a monoclonal band to reject the null hypothesis (Statistics performed by Richard Morris in the Department of Primary Care and Population Sciences, Royal Free Hospital).

Interestingly, patient A in the screening study reported here had Parkinson's disease and MGUS. As discussed above, *GBA1* heterozygosity is associated with an increased risk of idiopathic Parkinson's disease[352].

Patient B, diagnosed with myeloma, was found to be a compound heterozygote for N370S/R496H. However, she was in her 7th decade, had no visceral disease and Gaucher cells were absent on staging bone marrow examination. As described above, clinical penetrance is variable and many individuals, like patient B, will remain clinically silent unless investigated for an alternative co-morbidity. However, given the frequency of *GBA1* mutations in Ashkenazi Jews, all patients presenting with a paraprotein should be assessed for the possibility of GD. Currently, it is unclear whether GD patients with a paraprotein, who fail to meet traditional treatment criteria, should receive ERT[66]. However, as demonstrated in the RFH cohort of GD patients, with the exception of patient 8, ERT seems to prevent elevation in the

paraprotein level. This is keeping with the findings of Brautbar *et al.* (2004)[43]. Patient B remains untreated, for GD.

Patient 6, a N370S heterozygote, warrants further discussion, as he had IgD multiple myeloma and presented with a thoracic plasmacytoma. IgD is an infrequently expressed isotype in multiple myeloma with few case reports in the literature[398] and has been suggested to occur with a frequency of <0.5%[399]. IgD myeloma is associated with a poor prognosis and frequently presents with renal failure and amyloidosis[400]. This gentleman, however, remains in plateau since treatment in 2008. Whether the incidence of rare myeloma phenotypes, including IgD, IgE and non-secretory neoplasms, are increased in those with GD is unknown. Prior reports, including non-secretory IgD myeloma[156] and multiple hepato-splenic plasmacytomas[163] complicating GD, are present in the literature. However, rare presentations of plasma cells disorders, in GD, are more likely to be reported than those with more conventional presentations.

Patient 8 with AL-amyloidosis was found to be a carrier of an E326K mutation. It has been debated whether this *GBA1* allele is a polymorphism or a disease-causing mutation[401]. *GBA1* mutation analysis demonstrated the presence of E326K in 4 patients with GD (Type I, II and III; n=155)[401] but only in combination with an additional disease causing mutation in the same allele. E326K containing alleles in GD patients have been found to have an equivalent incidence as that found in a non-GD control population. A baculovirus expression system, using insect derived Sf9 cells, showed that the E326K allele led to an approximately 60% reduction in glucocerebrosidase activity compared to wild type[402]. Chabas *et al.* (2005) reported the frequency of E326K in a Spanish population of GD patients. They reported E326K to be present in approximately 40% of individuals with type II disease as part of a complex allele containing an additional mutation and speculated that it was a genetic modifier conferring increased disease severity. In the same expression system, the L444P/E326K allele led to lower enzyme activity than Sf9 cells expressing L444P alone[402]. Carriage of an E326K allele was not uncommon in a study screening for *GBA1* mutations in a predominately non-Jewish population of Parkinson's disease patients[375]. It has been hypothesised by Velayati *et al*. (2010) that mutant *GBA1* alleles, in the heterozygotic state, can lead to proteasomal

dysfunction secondary to ER stress[352]. It is unknown whether the E326K allele could lead to similar organelle dysfunction.

All Jewish patients with a paraprotein, that were identified to have *GBA1* mutations, were currently in at least their 7th decade of life. Therefore *GBA1* mutations seemed not to pre-dispose to monoclonal gammopathy at an earlier age. In contrast 4/10 GD patients in the RFH cohort developed a paraprotein prior to their 5th decade. Most individuals identified with a *GBA1* mutation had MGUS

In conclusion, this study continues to recruit, but currently lacks sufficient statistical power to refute the null hypothesis that carriage of a *GBA1* mutation pre-disposes to monoclonal gammopathy. Biomarkers of disease severity including chitotriosidase activity and serum ACE are higher in GD patients with gammopathy than those with normal immunoglobulins. These are macrophage-derived biomarkers and the hypothesis that lipid laden Gaucher cells support plasma cell growth and survival using cell culture-based models is further explored in chapter 4. Non-GD patients with plasma cell dyscrasias do not exhibit the same pattern of macrophage activation as that seen in GD. Alternatively, as disease severity predicts for gammopathy, other disturbances in innate or adaptive immunity, outside of the monocytic lineage, may confer an increased susceptibility to myeloma. This hypothesis is investigated in chapter 5. Currently, the International Gaucher Registry has inadequate data capture to record clinical information on GD patients with plasma cell disorders. By promoting the central collection of data pertaining to GD patients with myeloma or MGUS, several outstanding questions may be answered. These include:-

1. Should all GD patients with a paraprotein be treated with ERT?
2. Should all GD patients with myeloma be treated with ERT?
3. Do GD patients with myeloma respond to chemotherapy as well as those without GD?
4. Do patients with GD tolerate chemotherapy as well as those without GD?
5. Do GD patients on ERT with myeloma respond better to chemotherapy?
6. Is there a role for intensification of disease modifying therapy, including combined ERT/SRT treatment, in GD patients with multiple myeloma?

Although, there are several case reports speculating the answers to these questions, firm consensus and evidence is lacking.

4 *IN VITRO* MYELOMA CELL CO-CULTURE EXPERIMENTS

4.1 INTRODUCTION

The understanding of tumour biology in multiple myeloma has expanded over the past two decades, with a body of research focusing on the bi-directional relationship between plasma cells and the local bone marrow microenvironment. *In vitro* models have demonstrated a growth and survival advantage of primary or immortalised myeloma cells co-cultured on stromal or osteoclast monolayers[252;259]. Both macrophage and osteoclast cultures have been shown to protect chemotherapy treated myeloma cells from apoptosis[245;303]. Experimental models have also demonstrated an increase in osteoclastogenesis following co-culture with myeloma cells[302].

Skeletal disease, including osteopenia and lytic lesions, are common clinical findings in both GD and myeloma, suggestive of osteoclastic over-activity[28;403]. As demonstrated in chapter 3 and previously in the literature[158], patients with GD have large infiltrates of foamy macrophages abutting plasma cells on bone marrow examination. Macrophages have also been shown to be more prominent in the bone marrow trephines of non-GD patients with myeloma compared to those with MGUS, implicating a role for these monocytic-derived cells in the development of plasma cell dyscrasias[245]. The pathological mechanisms underlying the development of gammopathy in GD are unknown and likely to be multi-factorial. Abnormalities within the bone marrow environment, including macrophage accumulation, deposition of extracellular glucosylceramide, chronic immune stimulation, elevated B-cell proliferating cytokines and impaired tumour surveillance are all speculated to play a role[66;74;81;133;138;234;335]. The hypothesis explored in this chapter is that the GD bone marrow microenvironment is more permissive to plasma cell growth and survival compared to those without GD.

Chemoresistance is a challenge in the treatment of multiple myeloma. Currently it is not possible to predict which patients will respond to a particular chemotherapy regimen. Inter-individual differences in drug kinetics and acquisition of protective

mechanisms within the malignant cells themselves (drug efflux pumps, *p53* mutations) alter the sensitivity of malignant cells to chemotherapy[404]. Cytogenetic abnormalities including t(4;14), 17p-, t(14;16) and t(14;20) predict for an inferior treatment outcome to traditional chemotherapeutic agents. Translocations involving chromosome 14 couple the IgH enhancer gene with the fibroblast growth factor receptor 3 [FGFR3; t(4;14)], multiple myeloma SET domain gene [MMSET; t(4;14)], MAF oncogene [t(14;16)] and MAFB gene [t(14;20)][405]. Mechanistically, these cytogenetic aberrations enhance plasma cell proliferation and survival. The loss of chromosomal material on 17p- is associated with down-regulated *p53* activity (a tumor suppressor protein). Novel agents, including bortezomib and lenalidomide, have been suggested to overcome some of these poor prognostic variables including t(4;14)[405].

Additionally, the local bone marrow environment modulates chemoresistance, thus promoting research into the effects of anti-myeloma therapies on the non-tumour component. Thalidomide, lenalidomide and bortezomib have all been shown to inhibit osteoclastogensis[307-309]. The bisphosphonate zoledronate, an inhibitor of osteoclastogenesis, has been shown to alter survival in newly diagnosed patients with multiple myeloma (Morgan *et al*, European Haematology Association abstract book, Barcelona, 2010). *In vitro* studies have shown that zoledronate retards plasma cell growth and induces the apoptosis of both myeloma cell lines and IL-6 producing human stromal cells[406].

4.1.1 APOPTOTIC PATHWAY

Primary myeloma cells have a low proliferation rate and their accumulation over time may be secondary to a biological phenotype consistent with survival. Apoptosis is associated with a distinct pattern of morphological features, including nuclear condensation, membrane blebbing, chromatin clumping and the presence of an apoptotic body[407]. Apoptosis may be activated via either the intrinsic or extrinsic death pathways. Binding of TNFα, TRAIL or FAS ligand to their respective transmembrane death receptors activates the extrinsic pathway, resulting in the cleavage of caspase-8 (Figure 4-1). Bid, a pro-apoptotic protein, is cleaved by caspase-8 to a truncated form that then activates the intrinsic pathway[408].

Irradiation or chemotherapy leads to cell death mainly through the intrinsic pathway[409]. The intrinsic pathway, or mitochondrial-mediated pathway, is composed of a rheostat of regulatory proteins. The Bcl-2 family of related proteins includes both pro-apoptotic (Bid, Bik, Bim, Bax, Bak) and anti-apoptotic proteins (Bcl-2, Bcl-xL, Bcl-w and Mcl-1)[408]. These play a pivotal role in regulating the release of mitochondrial derived cytochrome *c*. Once a cell is committed to death, pro-apoptotic factors such as cytochrome *c* are released from mitochondria resulting in the activation of a cascade of caspases. Activation of effector caspases leads to the proteolytic cleavage of several proteins, including poly (ADP ribose) polymerase (PARP), DNA-dependent protein kinase and protein kinase C-delta in the terminal stages of cell death[407;408;410;411]. PARP is an 116kDa intra-cellular protein and is involved in DNA repair, which is heavily dependent on the consumption of ATP. In dying cells, where DNA damage is extensive, PARP is inactivated by proteolytic cleavage in order to preserve energy for adjacent cells that are either viable or salvageable from death.

Bcl-2, the first anti-apoptotic protein to be described, is over-expressed in follicular lymphoma due to the t(14;18) chromosomal translocation. Other lymphoid tumours, including multiple myeloma and diffuse large B-cell lymphoma (DLBCL) have been shown to over-express Bcl-2 in the absence of chromosomal rearrangements[412]. DLBCL has an inferior prognosis in the face of Bcl-2 over-expression[413]. Bcl-2 anti-sense therapy maybe of benefit in the treatment of patients with myeloma, chronic lymphocytic leukaemia and non-Hodgkin lymphoma[414]. The over-expression of Bcl-2 in multiple myeloma has been correlated with disease stage and may be a risk factor for extra-medullary disease[415;416]. Mcl-1, an anti-apoptotic protein, has been shown to be upregulated in malignant plasma cells, as demonstrated by flow cytometry, and is associated with an inferior event-free survival[417]. Levels of Bcl-xL did not correlate with clinical outcome in one study[418]. However, Bcl-xL expression, as assessed on trephine biopsy, predicted for an inferior response to induction chemotherapy and was more prevalent at relpase[419]. Linden *et al*. (2004) developed a transgenic mouse model that over-expressed Bcl-xL in the B-cell lineage, under the regulation of the kappa light chain enhancer. These mice had elevated serum immunoglobulin levels, foci of plasma cells within their bone marrow together with

hyaline casts in the kidneys[420]. Pro-apoptotic proteins such as Bim, PUMA, tBid, Bad and Noxa regulate the activity of many of the anti-apoptotic proteins involved in the mitochondrial death pathway, including Bcl-2, Bcl-xL and Mcl-1[421]. Phosphorylation of ERK via activation of transmembrane receptor tyrosine kinases leads to the down-regulation of Bim expression[422]. *p53* is a tumour suppressor protein and is encoded by a gene on the short arm of chromosome 17. It has a well established role in preventing malignancy and in addition, is a key regulator of apoptosis, DNA repair and cell cycling[423]. Chemotherapy can cause apoptosis of cancer cells by inducing the expression of *p53*.[411] *TP53* mutations in cancers, including myeloma, confer a poorer prognosis[424]. *p53* interacts with pro-apoptotic Bcl-2 family proteins, including Bax, PUMA and Noxa.

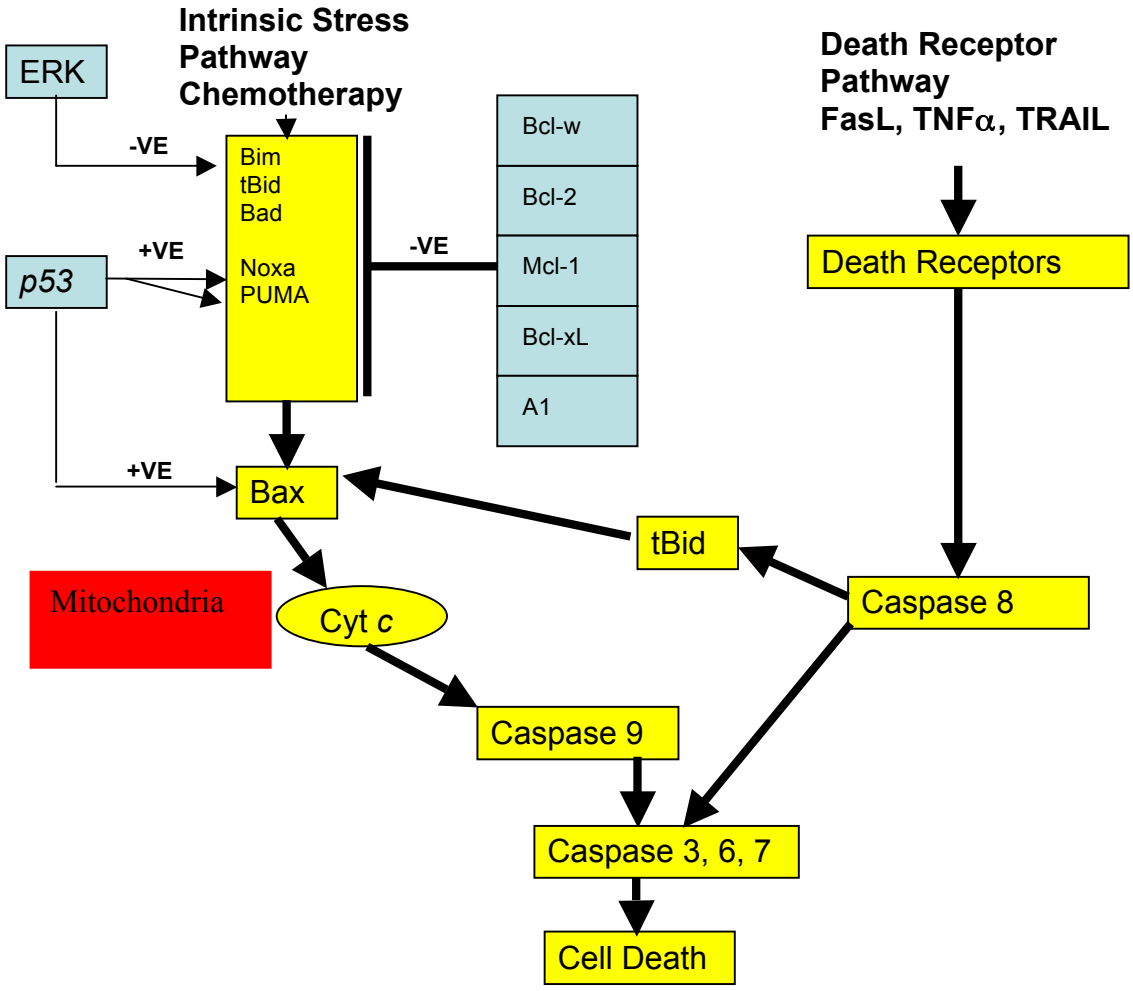


Figure 4-1. Pathways of apoptosis. Simplified schematic illustrating the intrinsic/mitochondrial death pathway and the extrinsic pathway, which is activated by death receptors.

4.1.2 HYPOTHESES

The following hypotheses are explored in this chapter:-

Hypothesis 1:- GD compared to non-GD derived monocytic cells preferentially support plasma cell expansion and survival.

Rationale:- Co-culture experiments in non-GD patients have demonstrated that osteoclasts and macrophages promote plasma cell survival. Osteoclast cultures have been shown to expand plasma cells.

Methods:- Plasma cell co-culture experiments utilising adherent monolayers derived from controls and GD patients.

Hypothesis 2:- GD compared to non-GD derived macrophages/osteoclasts protect myeloma cells from chemotherapy induced apoptosis.

Rationale:- Co-culture experiments of non-GD patients have shown that macrophages and osteoclasts reduce the sensitivity of plasma cells to chemotherapeutic drugs. Furthermore, there are anecdotal reports of poor responses to chemotherapy in GD patients with myeloma.

Methods:- Plasma cell co-culture experiments utilising adherent monolayers derived from controls and GD patients.

Hypothesis 3:- GD macrophages precursors are primed for osteoclastic differentiation.

Rationale:- Plasma cell co-culture promotes osteoclast formation in macrophage cultures derived from non-GD patients. Osteoclasts and plasma cells co-localise in the bone marrow specimens of non-GD patients and have been suggested to reciprocally facilitate the growth and survival of each other.

Methods:- Plasma cell co-culture experiments utilising adherent monolayers derived from controls and GD patients.

Hypothesis 4:- Compared to control cultures, GD derived macrophages/osteoclasts are more efficient at preventing apoptosis in myeloma cells pre-treated with chemotherapy.

Rationale:- Co-culture experiments in non-GD patients have shown that macrophages and osteoclasts rescue plasma cells pre-treated with chemotherapy.
Methods:- Plasma cell co-culture experiments utilising adherent monolayers derived from controls and GD patients.

4.2 METHODS

These methods are supplementary to those described in chapter 2.

4.2.1 D14-21 CO-CULTURE EXPERIMENTS

PBMCs, derived from both healthy controls and patients with GD, were isolated and plated ($0.25x10^6$ total) in 24-well plates as described in chapter 2. Each experimental well contained 3 glass inserts that were subsequently stained for TRAP following recovery on D21 (osteoclast enumeration). Cells were cultured in 1ml of osteoclast (OC) medium (R10, 25ng/ml M-CSF and 30ng/ml RANK-L) for the first 2 weeks with twice weekly changes in growth medium. On D14, $3x10^4$ NCI-H929 or $7.5x10^4$ U266 cells (minimum viability 90%) were added to co-culture wells or grown alone for a further 7 days in 3mls of OC medium. Three half medium exchanges (1.5mls) were performed during the final week of culture, including on D20. Plasma cells were recovered from duplicate experimental wells on D21 by re-suspending the non-adherent fraction by gentle agitation and washing twice in R10.

4.2.1.1 CONTACT DEPRIVED CO-CULTURE

Trans-well inserts were used in some experiments to deprive co-cultured plasma cells from contact with the underlying osteoclast monolayer. 0.4μm pore polyethylene terephthalate coated transwell inserts (Millicell; Millipore, Dundee, UK) were placed in 24-well plates. Myeloma cell lines were grown in the upper chamber. Co-cultured plasma cells were recovered by gentle re-suspension and washing three times with 1ml of R10. Bi-polar microscopy confirmed the absence of residual myeloma cells.

4.2.1.2 CO-CULTURE WITH ERT

Imiglucerase (Cerezyme™, Genzyme Inc., Cambridge, MA), enzyme replacement therapy (ERT), was added to Gaucher-derived osteoclast cultures on D1, at a concentration of 1unit/ml. Co-culture experiments were otherwise performed identically to that described above. Paired wells were cultured in the absence or presence of ERT until termination on D21. Aliquots of concentrated imiglucerase were stored at -80°C until required.

4.2.1.3 CO-CULTURE (D14-21) OUTCOME ASSAYS

Using the methodology described in chapter 2, plasma cells harvested from co-culture wells were subjected to GIEMSA staining, trypan blue staining, annexinV/PI immuno-staining (0.25-0.5x10^6 cells) and BrdU/PI analysis (0.5x10^6 cells). In addition, plasma cells were re-suspended at 0.22x10^6cells/ml and 90µl of cell suspension was plated in triplicate in 96-well plates. Cells were exposed to doubling dilutions of doxorubicin or melphalan for 24 hours before undergoing a 4-hour MTT dye reduction assay. Glass inserts, harbouring the adherent monolayer, were removed carefully with forceps and stained for TRAP as described in chapter 2.

4.2.2 RESCUE OF DOXORUBICIN TREATED U266 MYELOMA CELLS

Osteoclast cultures, GD or control-derived, were grown in 1ml of OC medium with two changes of medium per week until D18. U266 cells (0.5x10^6/ml) were pre-treated with 0µM, 0.5µM, 1µM, 2µM or 4µM doxorubicin for 6 hours, washed twice in PBS and re-suspended in 1ml of OC medium. Drug treated cells were plated on monolayers of osteoclasts that had been grown for 18 days or plated alone in 2mls of OC medium. U266 cells were then cultured alone or with osteoclasts between D18-21. All experimental conditions were performed in duplicate. Following this, non-adherent cells were harvested and counted, analysed by cytospin, stained with annexinV/PI (0.25-0.5x10^6 cells) or plated in a 4 hour MTT dye reduction assay (1x10^4 cells in triplicate).

4.2.3 PLASMA CELL PURITY DETERMINED BY GIEMSA STAINING

Cytospins were prepared from recovered cells by spinning 2.2x10^4 cells at 400rpm for 3 minutes. After drying for 2 hours, slides were fixed in methanol and stained for 30 minutes in 1:20 GIEMSA solution (Chapter 2). Cultured plasma cells were readily identifiable from primary mononuclear cells on D21. Myeloma cells (U266 or NCI-H929 cell line) have eccentrically placed nuclei, clumped chromatin, densely basophilic cytoplasm and a prominent peri-nuclear Golgi zone. Multi-nucleate plasma cells were often seen. In comparison, cultured macrophages were larger in size and had dusky grey cytoplasm with evident vacuoles. Macrophages also had an

eccentrically placed nucleus but the chromatin was less clumped in comparison to plasma cells (Figure 4-2). Purity was based on a minimum cell count of 200.

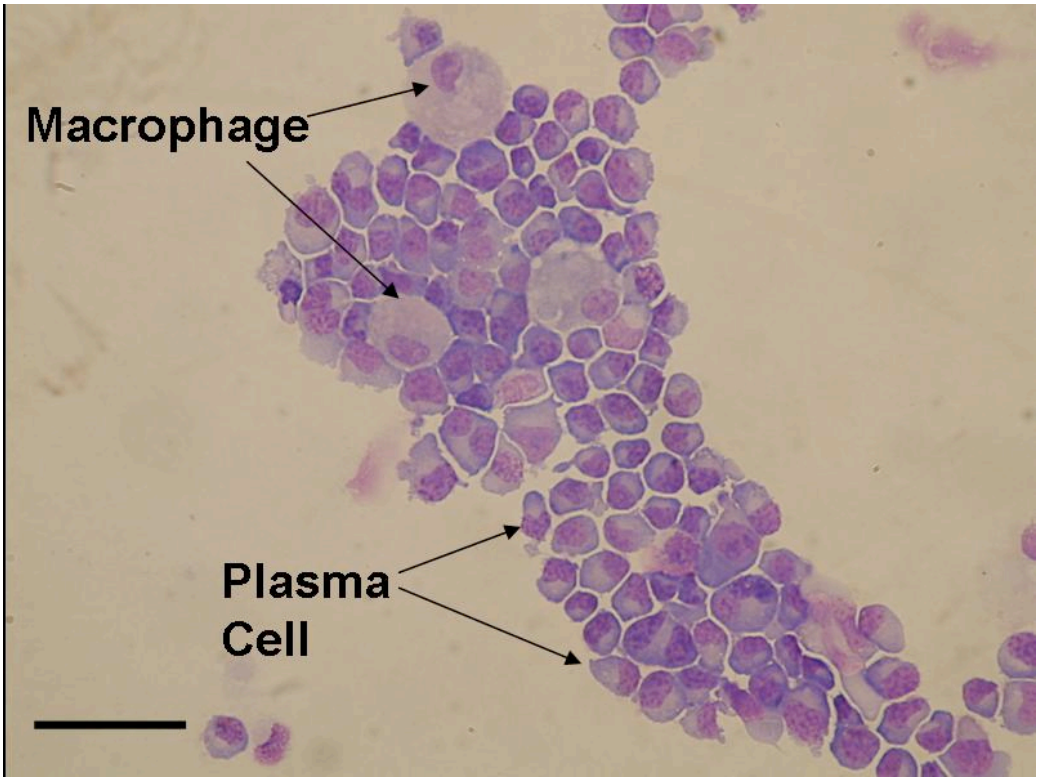


Figure 4-2. Plasma cell purity was determined morphologically from cytospins of harvested cells. Scale bar 100µm.

4.2.4 MACROPHAGE CYTOTOXICITY ASSAYS

$4x10^4$ peripheral blood monocytes (CD14+ve/64+ve) were plated in triplicate in 96-well plates and grown in OC medium. Growth medium was changed twice in week 1 and on alternative days between D8-18. On day 18, doubling dilutions of doxorubicin (0-16µM) and melphalan (0-200µM) were added for 48 hours prior to undergoing a 1 hour MTS dye reduction assay as described in chapter 2.

4.2.5 LYSATES FOR WESTERN BLOTTING

NCI-H929 myeloma cells ($4x10^4$ cells) were co-cultured on GD or control derived monolayers between D14-21 as previously described. Cells recovered from co-culture were incubated for 2 hours on tissue culture plastic in order to adhere out

contaminating monolayer cells. Plasma cell purity was determined by Giemsa staining. Protein lysates were made from duplicate co-culture wells derived from GD patients or healthy controls containing untreated NCI-H929 cells. In addition, lysates were made from plasma cells re-suspended in R10 and exposed to 50μM melphalan for 24 hours.

4.2.6 HARVESTING ADHERENT MONOLAYER CELLS

Adherent cells were washed in PBS for 15 minutes at 37°C. Aspirated PBS was replaced with a 1ml mixture of lidocaine and EDTA (100mls 0.02M EDTA solution, Sigma Aldrich: 10mls 2% lidocaine, Hameln pharmaceuticals) and returned to the cell incubator for a further 15 minutes. Cells were then recovered by gentle pipetting. Bi-polar microscopy confirmed the complete harvesting of the adherent cell fraction. Recovered cells were centrifuged at 300*g* for 10 minutes and re-suspended in R10. Cytospins were prepared. Cell suspension was stained with To-PRO-3 iodide and the number of apoptotic cells was enumerated by flow cytometry as described in chapter 2.

4.3 RESULTS

4.3.1 VALIDATION OF EXPERIMENTAL TECHNIQUES

Initially described in section 4.3.1 is data validating experimental techniques including:-

- The culture of macrophages/osteoclasts from GD patients and healthy controls (culture alone)
- Experiments validating and optimising the plasma cell/osteoclast co-culture model
- Assays employed assessing myeloma cell line purity, growth, survival and chemo-sensitivity in culture alone or post osteoclast/macrophage co-culture
- Confirmation of the glucocerebrosidase activity of monocytes derived from healthy controls and GD patients

4.3.1.1 OSTEOCLAST CULTURE

Monocytes derived from the peripheral blood of healthy controls and GD patients ($0.25x10^6$) were grown in osteoclast medium for 3 weeks. Adherent monocytes were initially small and spherical but by day 7 had become larger in size and developed spindle shaped morphology with polar cytoplasmic projections (Figure 4-3). After 21 days in culture, there was a mixture of spindle shaped cells and larger multi-nucleate forms with abundant cytoplasm. Many multi-nucleate cells exhibited ruffled edges and morphologically had the appearance of either mature macrophages or osteoclasts. GD cultures demonstrated a larger number of multi-nucleate cells at day 21 compared to controls. Osteoclasts were distinguished from multi-nucleate macrophages by demonstrating TRAP positivity on histo-chemical staining in both control and GD cultures. Both control and GD-derived monolayers were successfully grown until D21 without noticeable decreases in density or signs of toxicity, including cytoplasmic vacuolation, cell fragmentation and floating debris.

In conclusion, it was demonstrated, that peripheral blood monocytes were able to be cultured for 21 days and can differentiate into osteoclasts (see section 4.3.2).

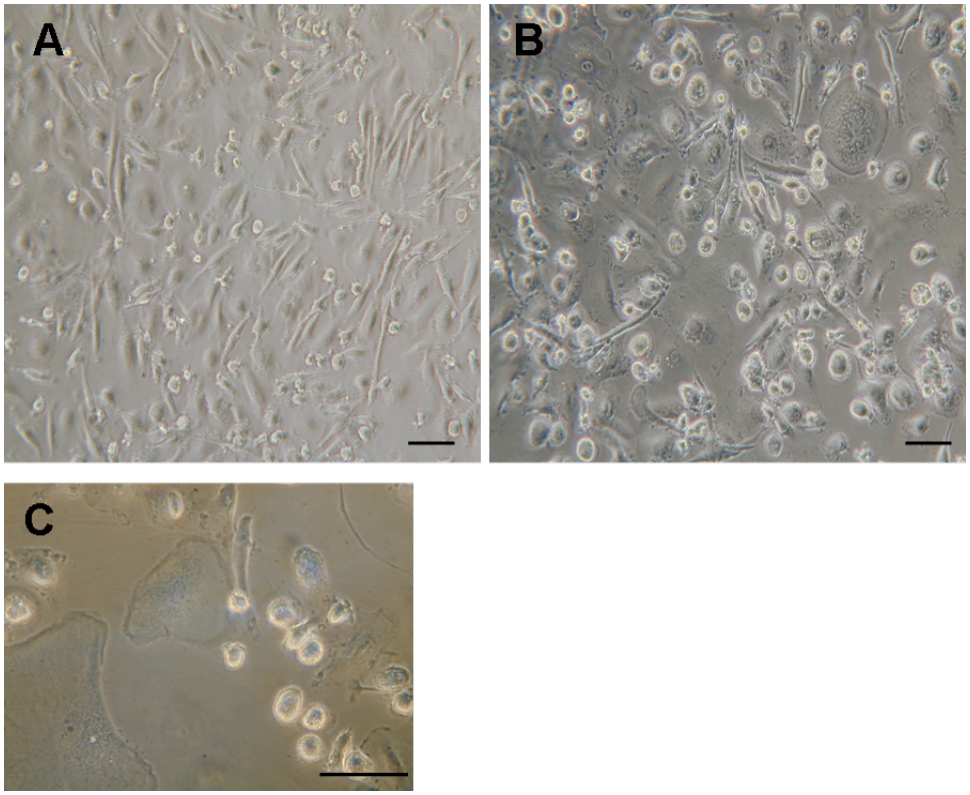


Figure 4-3. Microscopic appearance of cultured macrophages by bi-polar microscopy. Bi-polar microscopy demonstrating spindle shaped macrophages at day 7 (A) together with a mixture of larger cells with abundant cytoplasm post 21 day culture (B, C). Scale bar 40μm.

4.3.1.2 OPTIMISATION AND VALIDATION OF THE CO-CULTURE MODEL

As the co-culture model was developed *de novo*, many experimental modifications were required to address initial methodological problems. The following preliminary experiments were carried out in order to optimise the model:-

Harvestable Plasma Cell number

Approximately $3x10^6$ harvestable myeloma cells were required following co-culture in order to complete all out-come measures (cell count, trypan blue, cytospin, annexin/PI, BrdU/PI cell cycle and MTT assay). Prior to recovery, $0.1\text{-}1x10^5$ myeloma cells were plated for 7 days on osteoclast cultures between D14-21. Titration experiments demonstrated $3x10^4$ NCI-H929 or $0.75x10^4$ U266 plated cells respectively were required to yield the desired cell number from triplicate experiments following 7 days co-culture. No difference in recoverable cell number was observed between GD and control co-cultures.

Growth Medium Exhaustion

Co-culture experiments (D14-21) using a 1ml working volume led to medium exhaustion by D21 for both myeloma cell lines. Further experiments established a 3ml working volume with three 1.5ml medium changes between D14-21 as optimal. In addition, a residual volume buffer of >1ml during medium changes prevented the inadvertent aspiration of co-cultured myeloma cells (confirmed visually).

Bi-polar Microscopy

Myeloma cells were smaller in size and more spherical than the underlying adherent monolayer cells (Figure 4-4). Myeloma cells in the first few days of co-culture co-localised with or formed clusters over the underlying mononuclear cell culture. Following 7 days of co-culture, myeloma cell numbers had vastly increased, forming sheets of cells. There was no morphological difference, by bi-polar microscopy, between control and GD-derived monolayers following 7 days of co-culture. Cell densities over inserted glass coverslips were fairly uniform after 3 weeks incubation, irrespective of the origin of the mononuclear cells.

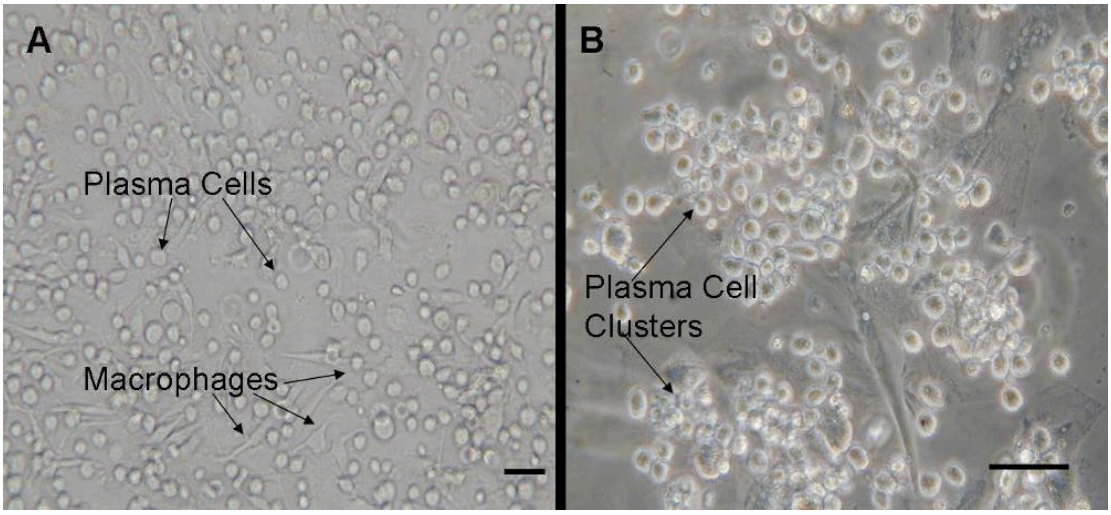


Figure 4-4. Bi-polar microscopy of co-cultured U266 myeloma cells on D21.
(A, B) In comparison to spindle shaped macrophages, U266 myeloma cells are small and form adherent clusters to the underlying monolayer. Scale bar 50µm.

4.3.1.3 PLASMA CELL PURITY AND CHEMOTHERAPY-INDUCED LOSS OF CD138 SURFACE EXPRESSION

This section investigates the identification, morphologically and by flow cytometry, of myeloma cell lines harvested from osteoclast co-culture. It is important to establish whether the cell surface expression of plasma cell markers (e.g. CD138) remains intact post co-culture or diminishes with chemotherapeutic insult. If the expression of plasma cell markers remains intact, then this can be employed to gate on the population in flow cytometric assays.

Both the U266 and NCI-H929 myeloma cell lines express CD138 (Sydecan-1) on their cell membranes. However, CD38, another common plasma cell marker, was absent on the cell surface of U266 cells (see section 2). Preliminary co-culture experiments were performed to assess CD138 expression as a surrogate marker of plasma cell purity. U266 cells were exposed to various concentrations of doxorubicin (0-4μM) for 6 hours, washed and plated either alone or in co-culture (D18-21) for 3 days. Plasma cells were identified based on low side scatter. In comparison, macrophages have a significantly higher side scatter profile.

Drug treatment led to loss of CD138 expression in plasma cells cultured either alone or in co-culture. Cells harvested from osteoclast wells at all drug concentrations had a higher percentage of CD138+ve cells compared to non-co-culture wells (Figure 4-5; Figure 4-6). Two-way ANOVA demonstrated both drug concentration ($p<0.001$) and co-culture ($p<0.01$) to have a significant effect on CD138 expression. The combination of a low percentage of CD138-ve U266 plasma cells in stock solution and the loss of expression with chemotherapeutic insult adds error in using this marker to assess for low levels of contaminating primary adherent mononuclear cells.

Therefore morphological determination of plasma cell purity following Giemsa staining was used as the preferred method. Additionally, plasma cell purity could be enriched by the 2 hour incubation of harvested cells on tissue culture plastic in order to adhere out contaminating macrophages or osteoclasts.

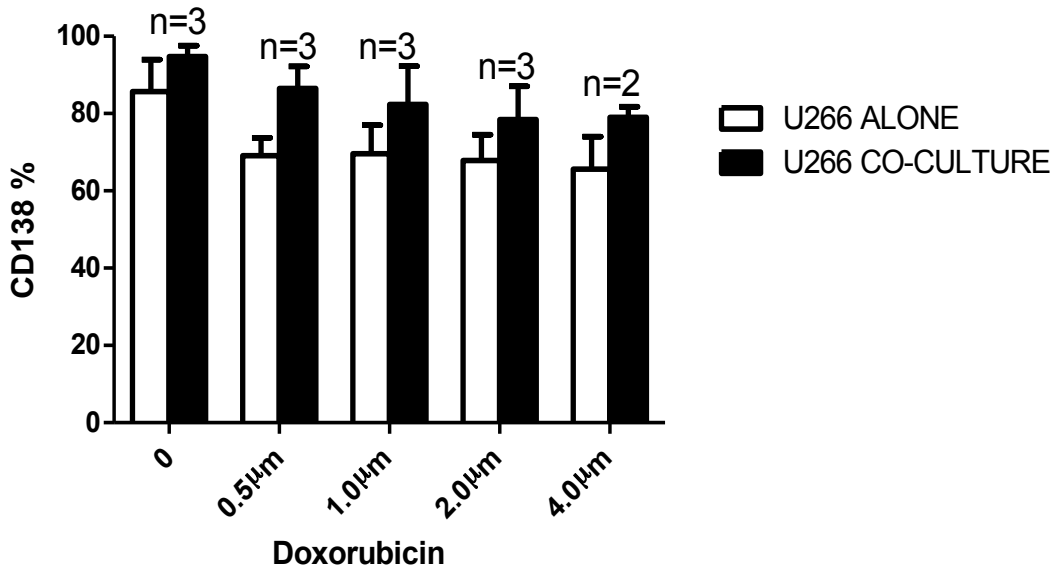


Figure 4-5. CD138 quantification of doxorubicin treated myeloma cells 72 hours post-treatment at stated concentrations.
Following drug exposure, plasma cells were incubated alone or on D18 osteoclast cultures. Mean+SD plotted.

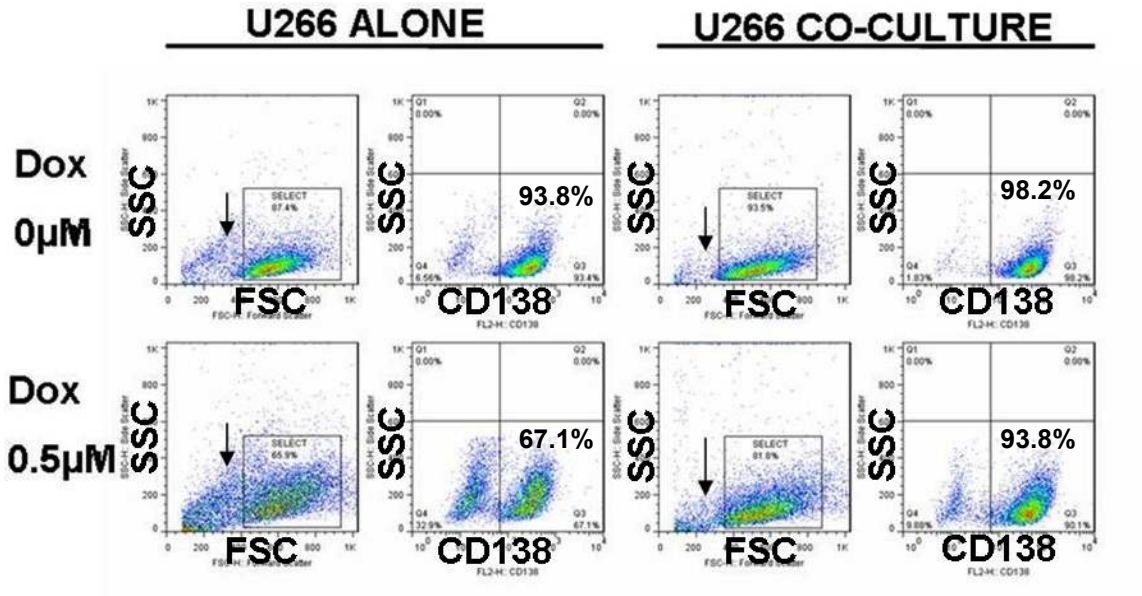


Figure 4-6. Representative flow cytometry plots of gated plasma cells harvested after 72 hour exposure to doxorubicin (Dox). Plasma cells were cultured alone or co-cultured with D18 osteoclast layers.
Arrows represent cell debris/necrotic cells. FSC (forward scatter), SSC (side scatter). Chemotherapy exposure leads to the loss of CD138 expression, which was ameliorated by co-culture.

4.3.1.4 FLOW CYTOMETRY AND MORPHOLOGICAL CHARACTERISTICS OF CO-CULTURED U266 CELLS

As described above, U266 plasma cells were pre-treated with doxorubicin and cultured alone or in co-culture (D18-21) for 3 days. More cell debris and necrotic cells (Figure 4-6; arrowed) were evident at lower forward scatter in doxorubicin treated U266 only wells compared to co-culture wells at the corresponding doxorubicin concentration. Co-cultured plasma cells had a lower and more uniform side scatter profile compared to U266 cells cultured alone.

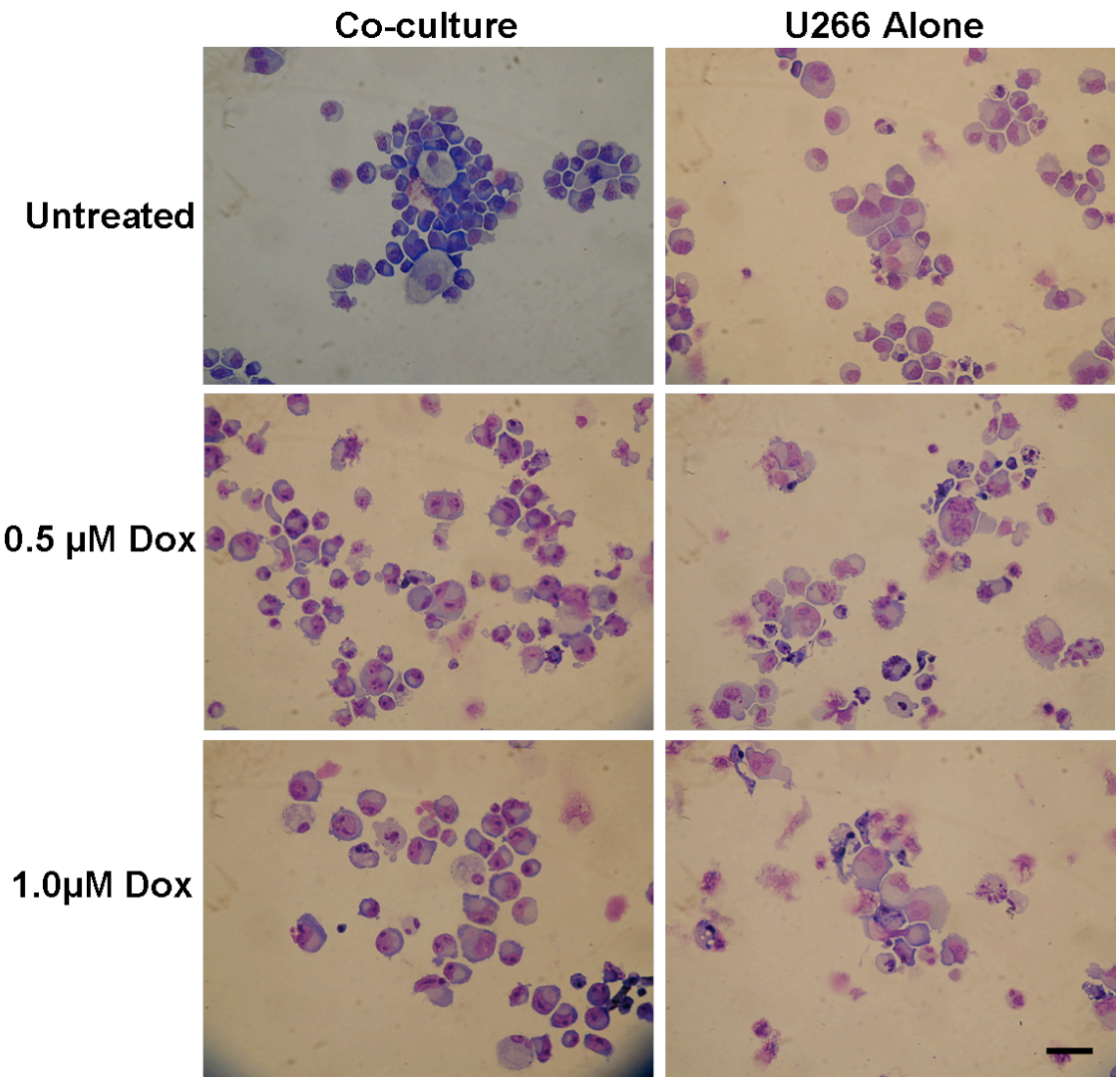


Figure 4-7. Representative cytospins of recovered U266 cells, untreated or pre-treated with doxorubicin (Dox), following 72 hour co-culture or culture alone. Scale bar 50µm.

These observations were confirmed morphologically by GIEMSA staining (Figure 4-7). Cytospins of doxorubicin treated U266 cells cultured alone showed significantly more apoptotic bodies compared to co-culture wells at all doxorubicin concentrations. In comparison to co-culture wells, doxorubicin treated U266 cells recovered from non co-culture wells were less healthy and contained cells with irregular cytoplasm, nuclear fragments, reduced basophilic staining and left-shifted plasmablastic morphology (visible nucleoli and non-clumped chromatin). These findings suggest that co-culture leads to a healthier recoverable plasma cell population and that the underlying monolayer may have phagocytic activity.

4.3.1.5 MYELOMA CELL LINE DRUG SENSITIVITIES

To establish drug sensitivities of myeloma cell lines for future co-culture experiments, U266 and NCI-H929 cells were treated for 72 hours with doubling dilutions of doxorubicin, melphalan, dexamethasone or bortezomib. Both myeloma cell lines were sensitive to melphalan, doxorubicin and bortezomib (Table 4-1; Figure 4-8). The U266 myeloma cell line was resistant to tested dexamethasone concentrations (61nm to 4mM). Therefore, dexamethasone was not used as a potential drug for future co-culture experiments. The therapeutic window for bortezomib, especially for the NCI-H929 myeloma cell line, was narrow, as demonstrated by the large decrease in absorbance with doubling drug concentration (Figure 4-8). Therefore melphalan and doxorubicin were selected as the drugs of choice for subsequent co-culture experiments.

	IC_{50} U266 (n=3)	IC_{50} NCI (n=2)
Doxorubicin μM	1.41±0.13	0.47±0.26
Melphalan μM	48.23±11.62	22.97±9.09
Bortezomib nM	8.04±6.75	16.24±13.55

Table 4-1. IC_{50} for doxorubicin, melphalan and bortezomib in the U266 and NCI-H929 cell lines. Mean and standard deviation indicated.

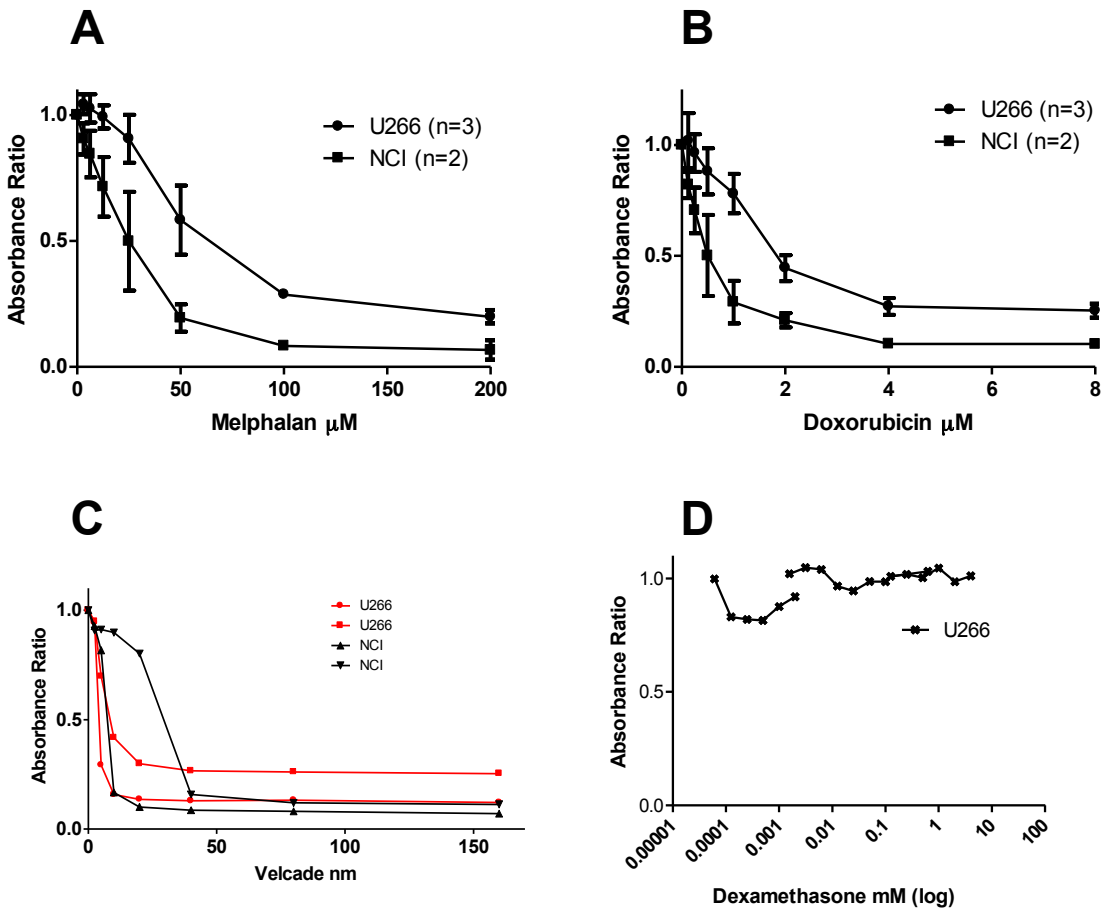


Figure 4-8. Drug sensitivity of the U266 and NCI-H929 myeloma cell lines to melphalan (A) and Doxorubicin (B).
Mean and SD plotted in (A) and (B). (C) Bortezomib, two drug cytotoxicity curves plotted for each cell line. The U266 cell line was resistant to dexamethasone at tested concentrations (D).

4.3.1.6 CALIBRATION OF ANNEXINV/PI ASSAY

U266 cells (0.5million/ml) were treated for 6 hours with doubling concentrations of doxorubicin and incubated for 72 hours prior to analysis. Healthy (annexin–ve, PI-ve), early apoptotic (annexinV+ve, PI-ve) and dead cells (annexinV+ve/PI+ve) were enumerated by flow cytometry. Increasing concentrations of doxorubicin led to a lower proportion of healthy cells and a higher proportion of apoptotic cells (Figure 4-9). A concentration-dependent increase in annexinV/PI+ve U266 cells was seen with increasing irradiation dose at 72 hours (data not shown). In conclusion, annexinV/PI quantification is a sensitive assay for assessing plasma cell viability following co-culture. In addition, doxorubicin concentrations greater than 1μM led to a significant increase (>20%) in the proportion of annexinV/PI positive cells. Therefore, doxorubicin concentrations greater than 1μm were used in subsequent co-culture experiments comparing the ability of the monolayer to rescue pre-treated U266 cells from apoptosis.

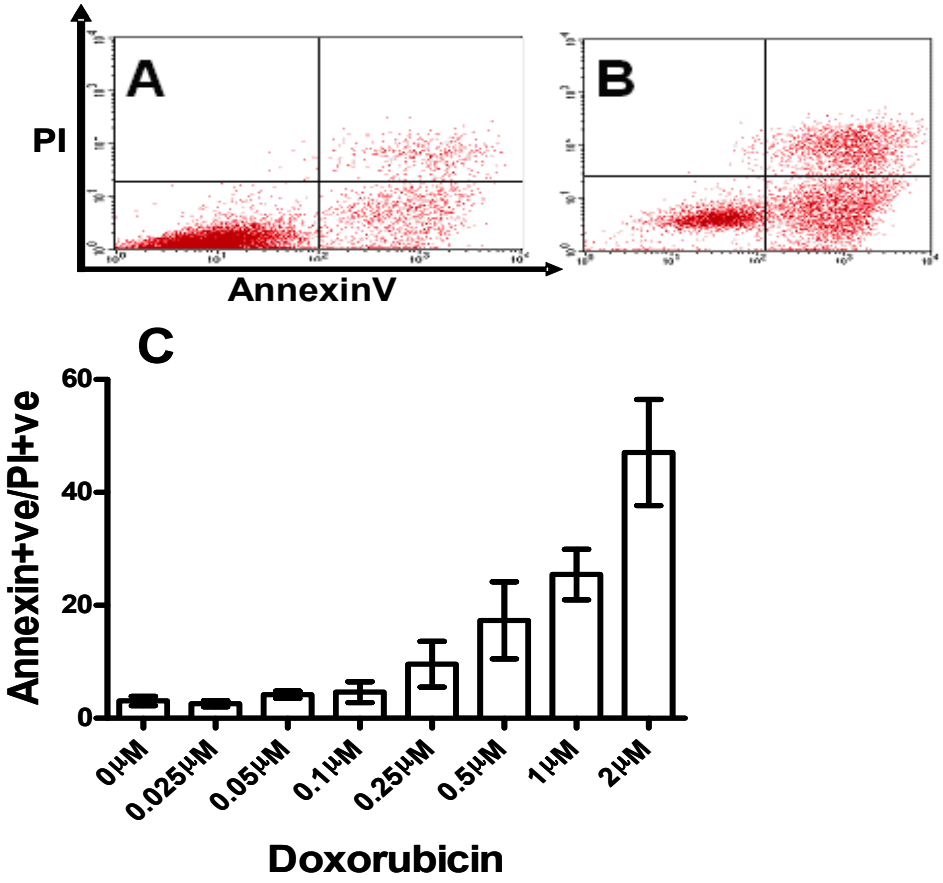


Figure 4-9. Annexin/PI quantification of doxorubicin treated U266 cells following 72 hour exposure.
(A) 0μM doxorubicin (B) 4μM doxorubicin. (C) Percentage annexinV/PI positive cells post exposure to various concentrations of doxorubicin (n=3; mean and SD plotted).

4.3.1.7 CALIBRATION OF BRDU/PI FLOW CYTOMETRIC ASSAY

BrdU is a thymidine analogue and is incorporated into the DNA of dividing cells during S-phase of the cell cycle, whereas PI is a nucleic acid stain. Primary plasma cells have a low proliferation fraction in contrast to immortalised cell lines that divide rapidly. Titration experiments were conducted to optimise the incubating concentration (10-40µM) and exposure time to BrdU (2-4 hours) for both the U266 and NCI-H929 cell lines. Both myeloma cell lines had greater than 30% of cells identifiable in S-phase following incubation for 4 hours with 20µM BrdU (optimised protocol), which was similar to the experimental protocol of others[425]. U266 cells ($0.25x10^6$) were treated with various concentrations of doxorubicin or irradiation for 6 hours and placed in a cell incubator for 72 hours. Increasing concentrations of doxorubicin and doses of irradiation led to a reduction in the proportion of cells in S-phase (Figure 4-10). It was concluded that this assay allowed sufficient sensitivity to assess the cell proliferation fraction.

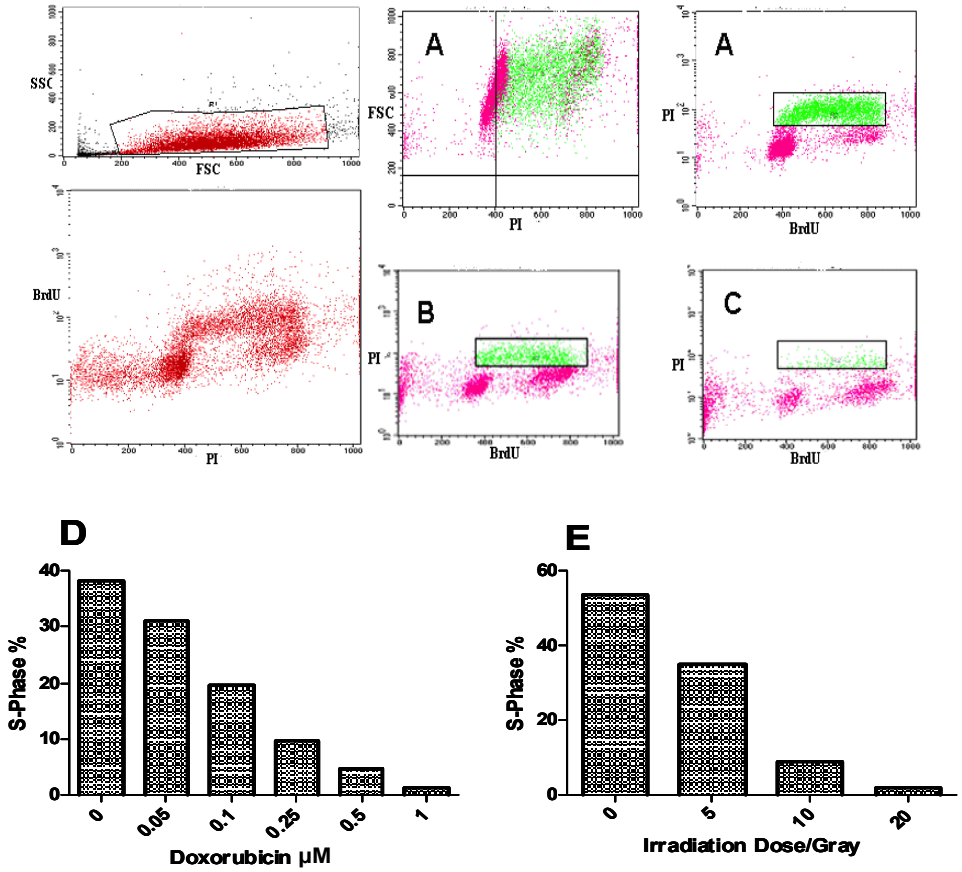


Figure 4-10. U266 cells exposed to (D) doxorubicin (n=1) or (E) irradiation (n=1) led to a concentration-dependent reduction in the number of U266 cells in S-Phase at 72 hours. Cell cycle plots demonstrate the number of cells in S-phase following 72 hour exposure to (A) 0µM doxorubicin, (B) 0.1µM doxorubicin and (C) 0.25µM doxorubicin.

4.3.1.8 CONFIRMATION OF CONTROL AND GAUCHER MONOCYTE GLUCOCEREBROSIDASE ACTIVITY

It was hypothesised that cultured monocytes, with abnormal sphingolipid metabolism, may preferentially support plasma cell survival and growth. Gated peripheral monocytes from GD patients had lower lysosomal glucocerebrosidase activity (Figure 4-11) than monocytes from healthy controls, as assessed by flow cytometry (see chapter 3). Monocyte glucocerebrosidase activity was determined by the MFI of liberated fluorochrome from the carrier substrate FDGlu and was expressed as a ratio of the sample MFI to that obtained from a paired specimen treated with the lysosomal glucocerebrosidase inhibitor CBE. GD derived PBMCs, based on morphological examination, were able to generate healthy monolayers after 21 days in culture with identical cell densities to those derived from control PBMCs.

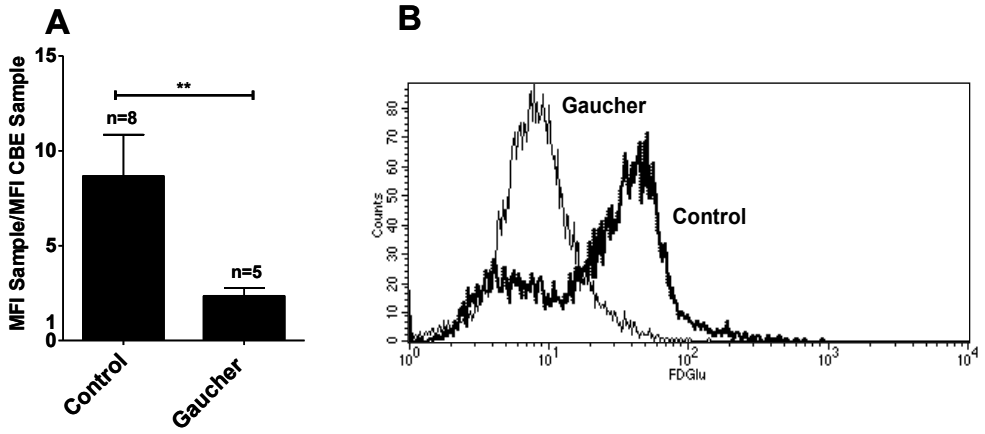


Figure 4-11. Monocyte glucocerebrosidase activity was determined by the MFI of liberated fluorochrome from the carrier substrate fluorescein di-β-D-glucopyranoside. Activity reported as a ratio of MFI Sample/MFI CBE Sample. Mean+SD plotted. **p<0.01

4.3.1.9 CONFIRMATION OF LYSOSOMAL DYSFUNCTION IN GD MONOCYTES

Immunofluorescent staining was used to assess the distribution of BODIPY lactosylceramide (visualised green) within the cell and the endo-lysosomal compartment was visualised red (Lysotracker). In monocytes derived from healthy controls, lactosylceramide was concentrated in the cellular membrane (Figure 4-12). GD monocytes accumulated lactosylceramide in their lysosomal compartment, as demonstrated by the co-localisation of BODIPY (visualised yellow). Therefore monocytic precursors plated from patients with GD had evidence of lysosomal dysfunction

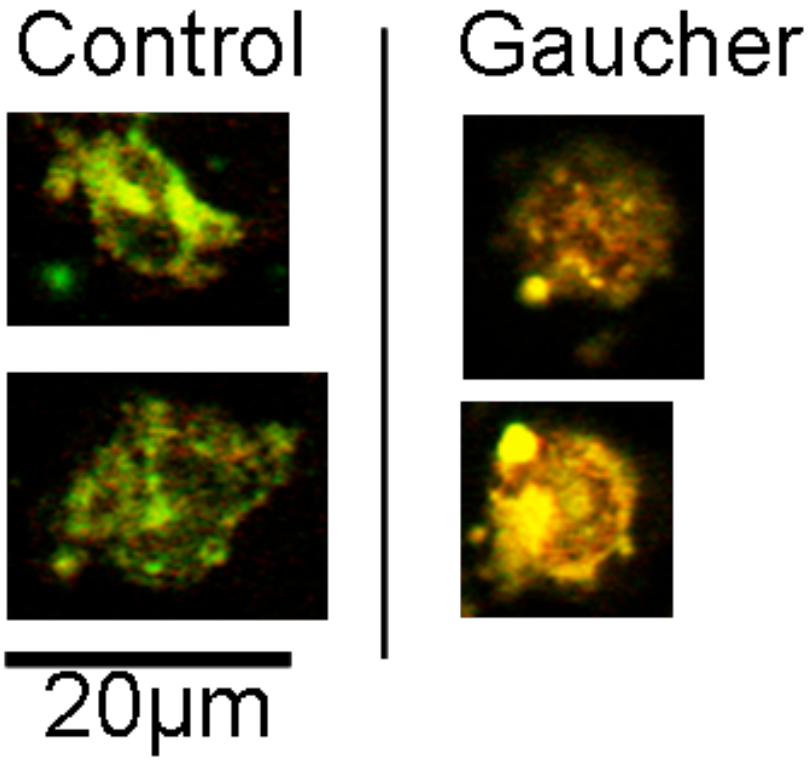


Figure 4-12. Representative examples of GD and control monocytes stained for lactosylceramide (green) and the lysosomal compartment (lysotracker; red).
GD monocytes due to lysosomal dysfunction accumulated lactosylceramide in the lysosomal compartment. The signals in control monocytes remain mostly separated.

4.3.2 OSTEOCLAST GENERATION FROM NON CO-CULTURE

It was demonstrated above that GD monocytes have low glucocerebrosidase activity and abnormal lipid trafficking. This section explores the ability of GD monocytes to undergo osteoclastic differentiation. Osteoclasts are known to support plasma cell growth *in vitro*.

After 21 days of culture, the number of TRAP+ve cells derived from controls or GD patients was enumerated (average of 2 coverslips). Cultures with sub-optimal density or morphological evidence of cell toxicity at day 21 were discarded. TRAP+ve cells with 3 or more nuclei were counted as osteoclasts. Cells that were TRAP+ve and contained less than 3 nuclei were labelled as pre-osteoclasts. Controls harboured a lower number of osteoclasts (8.1±6.8; Mean±SD) compared to GD patients (54.2±48.07; Figure 4-13) at day 21. Osteoclasts derived from GD patients were

generally larger in size than those seen in control culture with many being greater than 400μm in length. There were areas of both dense osteoclastogenesis and sparse osteoclast generation on recovered glass inserts from GD cultures (Figure 4-14). TRAP+ve cells generated from GD patients were generally of a higher ploidy than those seen in control cultures. Although not formally enumerated, the number of TRAP+ve cells with less than 3 nuclei was clearly less in controls compared to GD patients.

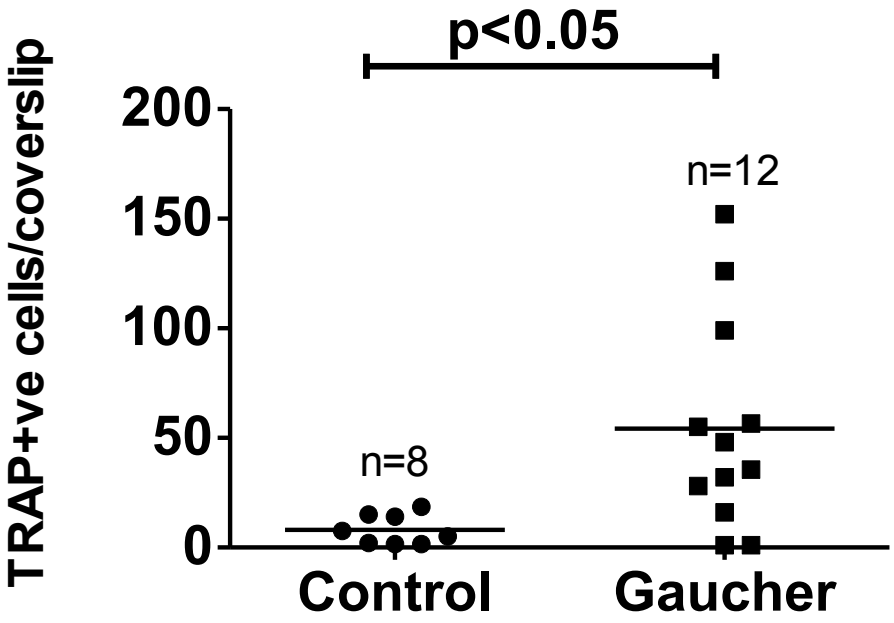


Figure 4-13. Number of osteoclasts generated following 21 day culture of adherent mononuclear cells from Gaucher patients compared to controls.
Mean=plotted line.

There was significant heterogeneity in the number of osteoclasts generated between Gaucher patients. In contrast, the number of osteoclasts generated from controls was uniformly low. This is a repeat observation of previous work from our laboratory pending publication (Mathew Reed, Dr Derralynn Hughes). In addition, our group has correlated the number of TRAP+ve cells with positive staining for the osteoclast vitronectin receptor and enhanced bone resorption. Gaucher disease leads to lytic bony lesions and osteoporosis, both indicative of osteoclast over-activity. This model has been optimised, both in culture duration and cytokine supplementation (RANK-L/M-CSF), to exploit theoretical differences in osteoclast generation between the GD and control bone marrow microenvironment. This model was used in subsequent co-culture experiments with plasma cells.

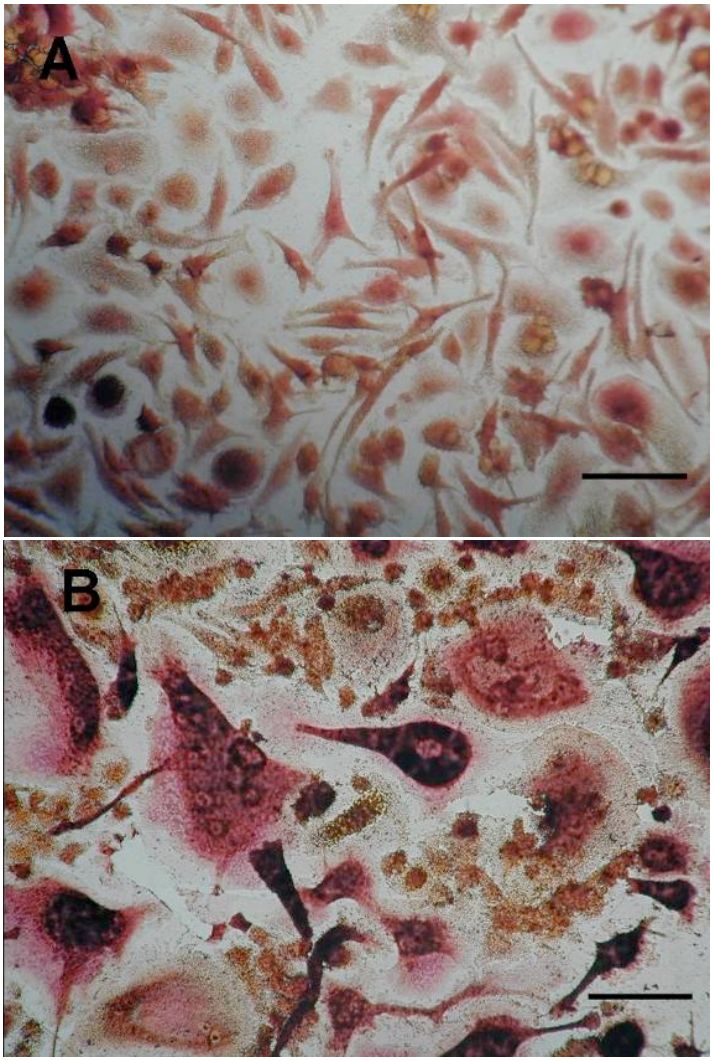


Figure 4-14. TRAP stain following 21 day culture of adherent mononuclear cells from controls and GD patients.
Sparse osteoclastogenesis is typical in controls (A). GD patients generate significantly more TRAP+ve osteoclasts and pre-osteoclasts in culture (B). Scale bar 100µm.

4.3.3 PLASMA CELL CO-CULTURE EXPERIMENTS (D14-21) PROLIFERATION/SURVIVAL

It has been demonstrated so far, that GD monocytes, have evidence of lysosomal dysfunction and more readily undergo osteoclastic differentiation than control monocytes. Experiments, from here in, explore whether these abnormalities, confer a proliferative or survival advantage on co-cultured myeloma cell lines.

Plasma cells proliferate when co-cultured with osteoclasts or macrophages. NCI-H929 cells ($3x10^4$) or U266 ($7.5x10^4$) cells were co-cultured with control or GD monolayers (D14-21). Plasma cells were co-cultured in contact with the underling

monolayer (CT) or trans-well inserts (TW) for 7 days. Harvested cells were counted and cell cycle analysis and viability testing were performed.

4.3.3.1 PLASMA CELL PURITY

Mean plasma cell purity, as determined morphologically by Giemsa staining, was greater than 94% for NCI-H929 or U266 cells co-cultured in contact. There was no statistical difference in mean plasma cell purity between controls and GD co-cultures (Table 4-2).

	Control	Gaucher
NCI-H929	95.5±2.2 (n=16)	95.7±3.1 (n=18)
U266	94.3±1.1 (n=8)	94.0±2.7 (n=8)

Table 4-2. Plasma cell purity of co-cultured plasma cells in a contact-dependent system. Mean±SD.

4.3.3.2 NCI-H929 PLASMA CELL NUMBERS

Contact-dependent cell culture, irrespective of monolayer origin, led to an increase in the number of harvested NCI-H929 plasma cells (Figure 4-15).

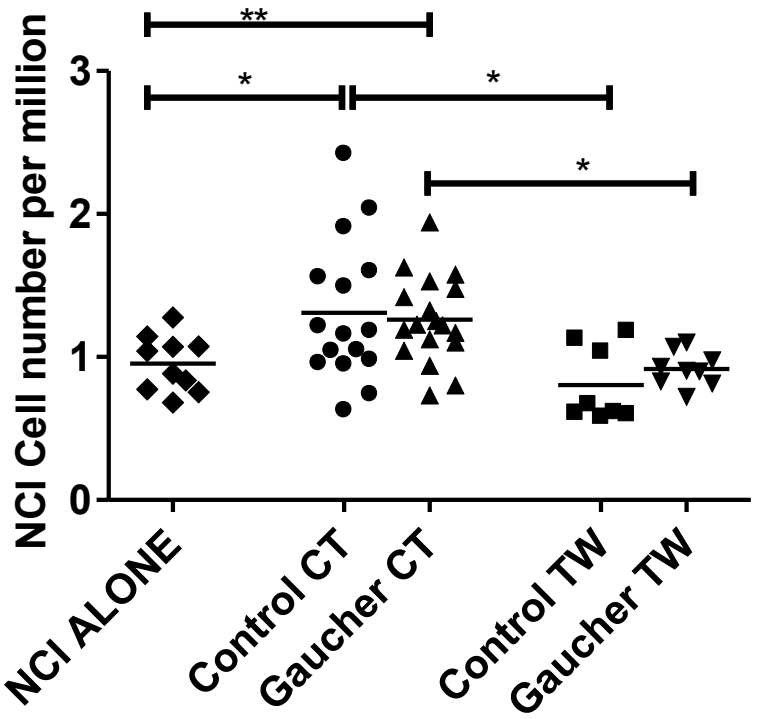


Figure 4-15. Number of NCI-H929 plasma cells harvested from contact co-culture (CT) or contact-independent co-culture (TW) compared to plasma cell only wells. Mean plotted. *p<0.05 **p<0.01.

There was no difference in the number of plasma cells harvested following contact co-culture between control ($1.3x10^6$±0.5; Mean±SD; n=16) and GD monolayers ($1.3 x10^6$±0.3; n=18). Although contact co-culture did lead to a statistical increase in NCI-H929 cell number, this was less than a 50% difference from the mean of plasma cell alone wells. In addition, there was significant heterogeneity in cell number between individual cultures. However, paired experiments, using trans-well inserts, confirmed the contact-dependent increase in NCI-H929 myeloma cell number (Table 4-3).

	NCI Contact	**NCI Trans-well**	**P Value**
Control	1.8±0.4(n=6)	0.87±0.3 (n=6)	p<0.05
Gaucher	1.3±0.4(n=9)	0.9±0.1 (n=9)	p<0.05

Table 4-3. NCI-H929 plasma cell number ($x10^6$) from paired co-culture experiments. Mean±SD. Wilcoxon Signed Rank Test.

Interruption of cell contact with the underlying monolayer led to a reduction in NCI-H929 plasma cell number to values seen post culture of NCI-H929 cells alone. There was no statistical difference between the number of NCI-H929 plasma cells recovered from contact deprived co-cultures of controls ($0.8x10^6$±0.26; Mean±SD; n=16) compared to contact deprived GD co-culture ($0.9 x10^6$±0.1; n=18) in this additional series of experiments.

4.3.3.3 U266 PLASMA CELL NUMBER

There was no significant difference between the number of U266 cells harvested from culture alone ($0.83x10^6$±0.18; Mean±SD; n=10) and contact co-culture with either GD ($0.77x10^6$±0.2; n=9) or control monolayers ($0.97x10^6$±0.14; n=8). Based on the increase in NCI-H929 plasma cell number in contact culture, reported here, and prior literature documenting the increase in U266 cells with osteoclast co-culture, this was an unexpected result. Giemsa staining of glass inserts from co-culture wells revealed large numbers of un-recovered plasma cells adhering to the underlying monolayer; this was despite two washes in R10 medium and gentle pipetting. More vigorous pipetting, however, led to a drop in plasma cell purity due to admixed monolayer cells. Co-culture wells with <90% purity were discarded from analysis. GD monolayers did not lead to a proliferative advantage over control

monolayers, based on the number of harvested cells, and in fact were statistically less ($p<0.05$).

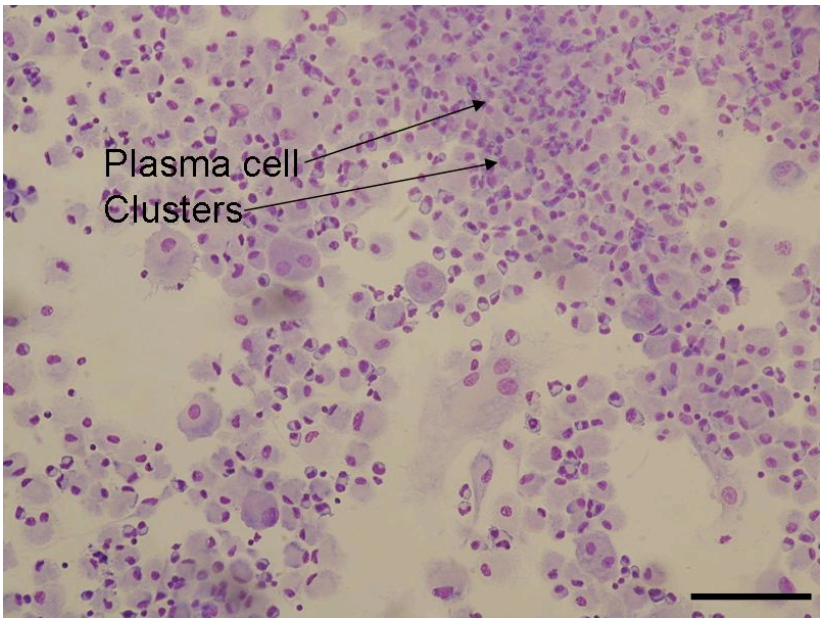


Figure 4-16. Giemsa stain of a recovered glass inserts from U266 control co-culture. A cluster of small plasma cells are seen co-localised with adherent GD or control monolayer cells. Scale bar 200µm.

4.3.3.4 CELL CYCLE ANALYSIS

Based on recoverable cell number, GD monolayers did not promote a proliferative advantage over control co-culture. However, U266 cells were unable to be fully harvested from co-culture wells due to adherence to the underlying monolayer (Figure 4-15). This is in contrast to NCI-H929 cells that were easily recoverable. Based on cell number alone, a proliferative advantage may have been masked if GD monolayers were more adhesive to U266 plasma cells then control monolayers. As an additional measure of cell proliferation, independent of cell adhesion, cell cycle analysis was performed on harvestable U266 or NCI-H929 cells grown alone for 7 days or following co-culture (D14-21). BrdU/PI analysis was used to calculate the percentage of harvested plasma cells in S, G0/G1 and G2/M phases of the cell cycle (Figure 4.17; Figure 4.18). Co-culture compared to culture alone did not lead to an increase in the number of plasma cells in S-phase for either the U266 or NCI-H929 cell lines. GD-derived co-culture compared to control co-culture did not lead to a higher percentage of U266 or NCI-H929 plasma cells in S-phase. There was an increase in the percentage of NCI-H929 cells in G0/G1 phase ($p<0.05$) from GD co-

culture compared to culture alone. Otherwise, cell cycle analysis for the different phases was similar between GD and control derived co-cultures.

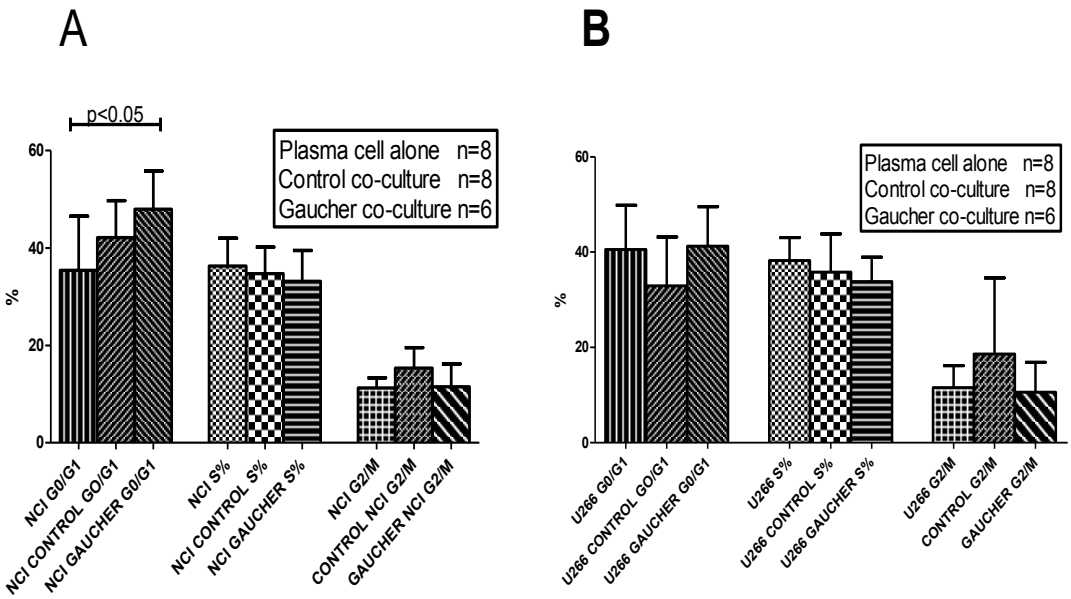


Figure 4-17. BrdU/PI cell cycle analysis of harvested U266 or NCI plasma cells following 7 day co-culture or culture alone. Mean±SD plotted.

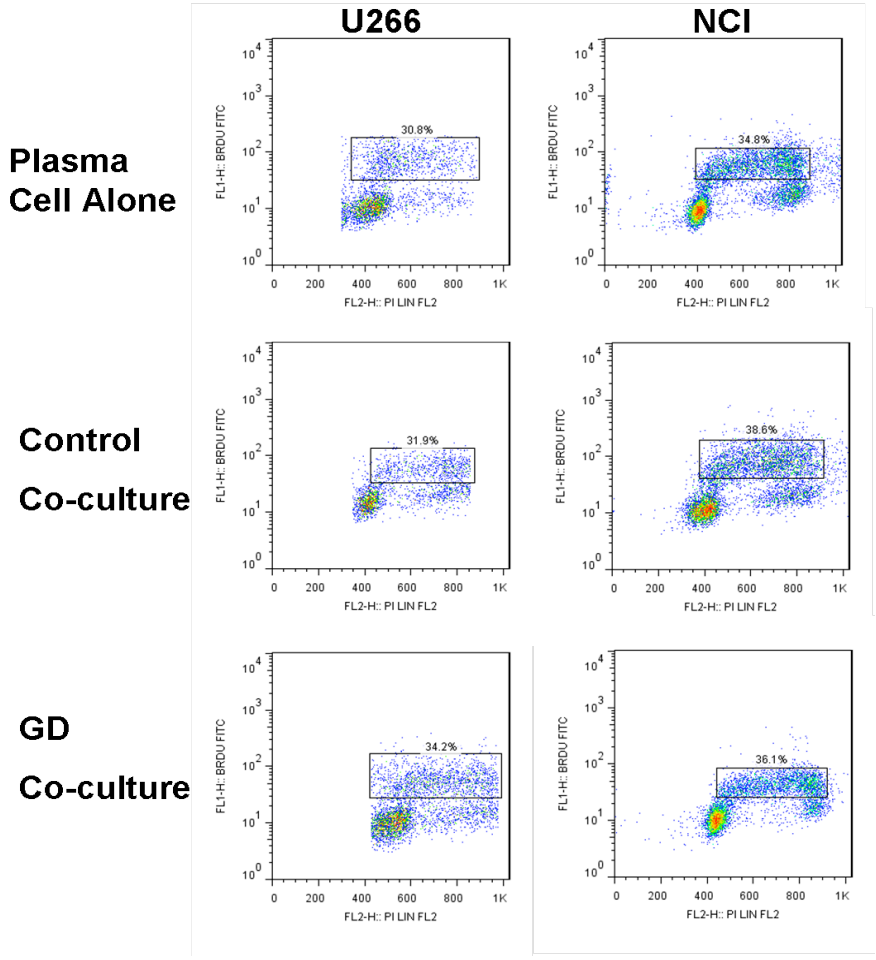


Figure 4-18. Representative BrdU/PI analysis of the % of cells in S-phase from plasma cell alone or co-culture wells (Control or GD).

4.3.3.5 PLASMA CELL SURVIVAL/VIABILITY

The viability of NCI-H929 or U266 cells following 7 days of co-culture or culture alone was assessed by trypan blue exclusion or annexinV/PI quantification (D14-21). Plasma cells were cultured in contact (CT) or physically separated from the underlying adherent monolayer by trans-well inserts (TW). All datasets represent a minimum of n=8, apart from contact-independent co-cultures derived from controls with U266 cells where n=5.

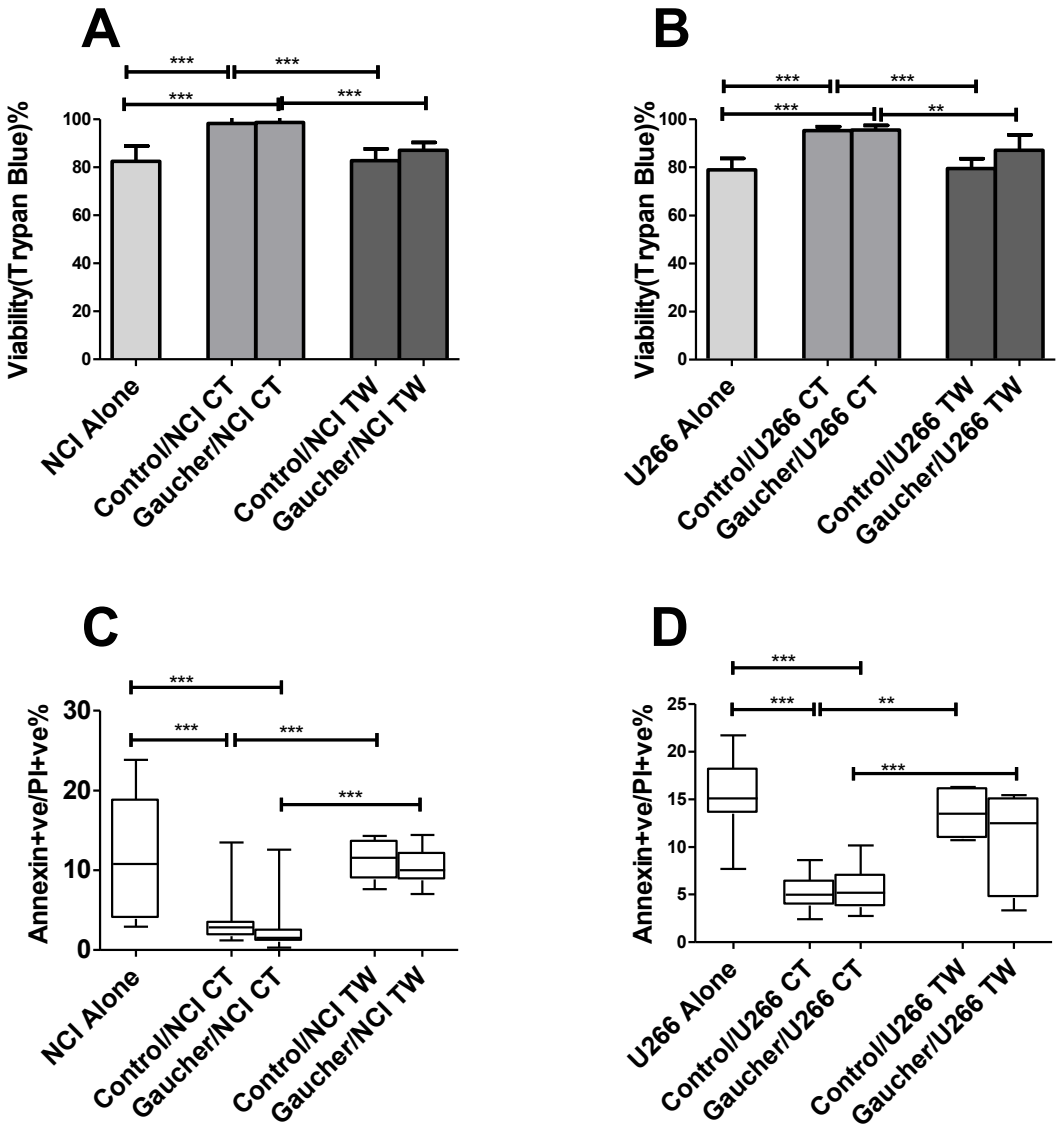


Figure 4-19. Viability of NCI-H929 and U266 cells lines determined by trypan blue exclusion (A,B) and AnnexinV/PI assay (C,D) following culture alone or co-culture.
Mean±SD plotted. Cell survival demonstrated by dual positivity for annexinV/PI or trypan blue exclusion. Box and whiskers plots in (C) and (D) represent median, inter-quartile range and maximum/minimum data values. Contact co-culture (CT), trans-well co-culture (TW). *p<0.05 **p<0.01 ***p<0.001

Co-cultured plasma cells, in a contact-dependent model, were more viable than those cultured alone based on both trypan blue exclusion and dual positivity for annexinV/PI (Figure 4-19; Figure 4-20). GD monolayers did not convey an additional survival advantage over non-GD derived co-cultures.

Contact-independent co-cultures using trans-well inserts led to a fall in plasma cell viability compared to contact co-culture for both the U266 and NCI-H929 myeloma cell lines. GD monolayers also did not confer a survival advantage over osteoclast cultures derived from controls, in transwell experiments. Compared to U266 plasma cell alone wells, non-contact GD co-culture led to a statistically lower percentage of annexinV/PI positive cells ($p<0.05$), raising the possibility of a soluble survival factor.

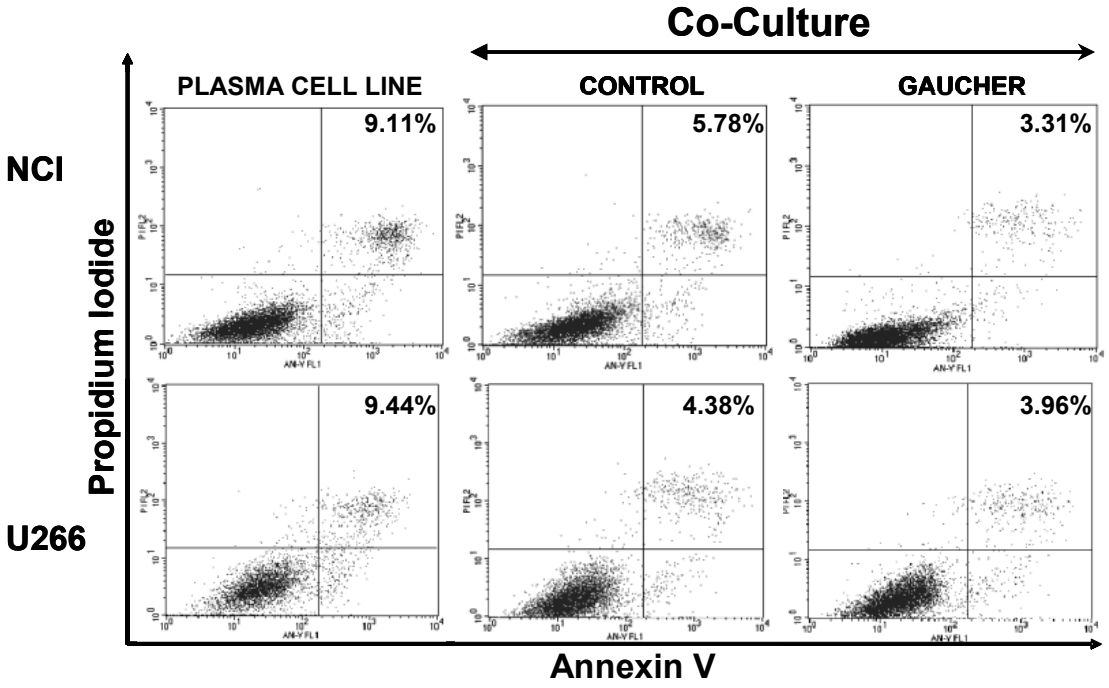


Figure 4-20. Representative flow cytometry plots of annexinV/PI immuno-staining of NCI-H929 or U266 plasma cells grown alone or in contact co-culture.
The figure in the upper right quadrant indicates the % of annexinV/PI positive cells.

4.3.4 DRUG SENSITIVITY OF CO-CULTURED PLASMA CELLS

4.3.4.1 MTT DRUG SENSITIVITY

Plasma cells were cultured alone or with control or GD-derived monolayers (D14-21) before being plated, in triplicate, in 96 well plates ($2x10^4$/well). Cells were exposed to doubling dilutions of melphalan and doxorubicin for 24 hours before performing a MTT dye reduction assay. Any chemo-protective advantage imparted on recovered plasma cells from co-culture is likely to diminish over time and therefore the drug IC_{50} was established at 24 hours rather than at a later time point. Both the U266 and NCI-H929 cell lines demonstrated a higher IC_{50} to either drug than that stated in the validation experiments above (section 4.2.1.5). This is explained by the fact that the MTT assay was performed at 72 hours in validation experiments rather than at 24 hours post co-culture. Additionally, drug sensitivities in section 4.2.1.5 were established in R10 medium whereas plasma cells were grown for 7 days in OC medium (RANK-L/M-CSF) in the experiments described here.

Control co-culture did not lead to an increase in the melphalan IC_{50} for either the NCI-H929 (NCI alone 44.9±14.8; Control/NCI CT 41.6±6.6μM; Mean±SD) or U266 cell lines (U266 alone 82.9±39.2 μM; Control/U266 CT 78.2±21.2 μM) compared to plasma cell culture alone (Figure 4-21). Gaucher co-culture, however, led to a significant increase in the melphalan IC_{50} of both the NCI-H929 (Gaucher NCI/CT 54.7±13.9 μM; $p<0.01$) and the U266 (Gaucher U266/CT 110.9±36.6μM; $p<0.01$) cell lines compared to control co-culture.

The doxorubicin IC_{50} for both myeloma cell lines in co-culture was not increased compared to non co-culture (Table 4-4).

	PLASMA CELL ALONE	CONTROL CO-CULTURE	GAUCHER CO-CULTURE
U266	2.3±0.9 (n=10)	2.3±0.9 (n=8)	3.3±1.4 (n=9) *
NCI-H929	1.9±0.9 (n=9)	1.9±1.1(n=14)	1.7±0.6 (n=18)

Table 4-4. Drug sensitivity to doxorubicin (μM), at 24 hours, of harvested (A) NCI or (B) U266 cells cultured alone or in co-culture (D14-21) for 7 days.
Mean±SD reported. *p=0.08

From these initial observations, GD monolayers seem to prime myeloma cell lines for survival against melphalan but not doxorubicin. A trans-well system was used to investigate whether the increase in melphalan IC_{50} was due to protection conferred by contact or due to secretion of a survival factor from the underlying GD adherent monolayer. Contact deprivation led to a decrease in the melphalan IC_{50} of NCI-H929 cells in paired GD co-cultures (contact IC_{50} 48.2μM; transwell IC_{50} 34.9 μM; paired t-test $p<0.01$;Figure 4-22). In addition, IC_{50} of melphalan in U266 cells was lower in cells harvested from trans-well inserts than co-culture in 3 out of 4 paired experiments using GD derived adherent monolayers (Figure 4-23).

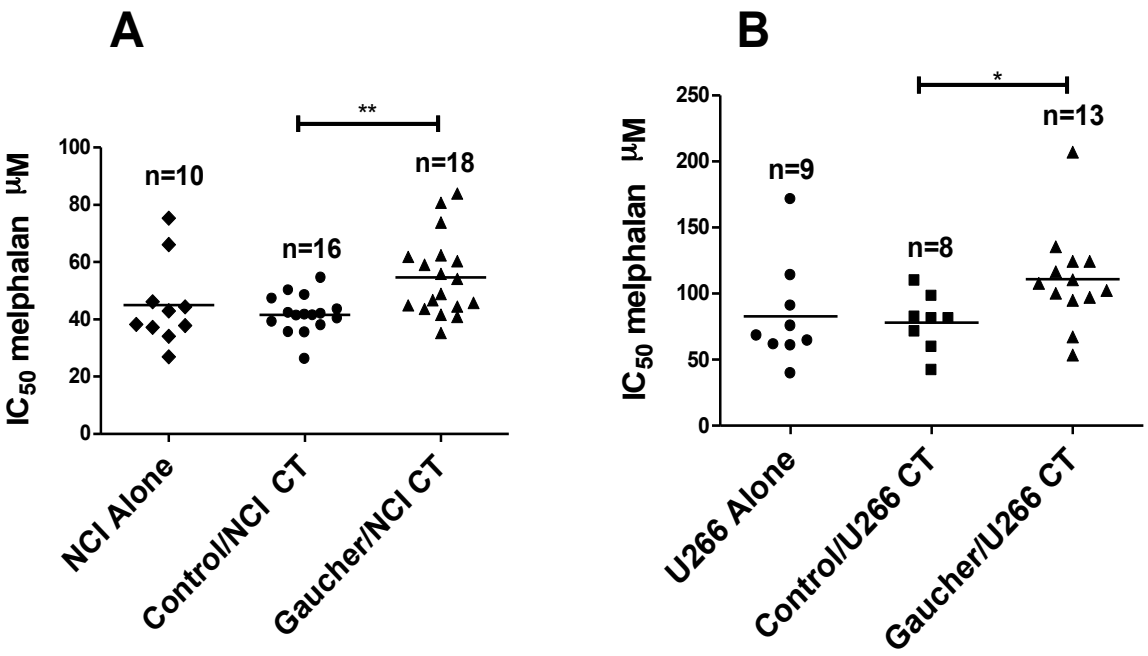


Figure 4-21. Drug sensitivity to melphalan of harvested (A) NCI-H929 or (B) U266 cells cultured alone or in co-culture for 7 days.
Contact co-culture (CT). Mean indicated by line. *p<0.05 **p<0.01

In contrast to GD cultures, NCI-H929 cells recovered from contact or contact-independent control co-cultures demonstrated identical chemosensitivity to melphalan at 24 hours. Disruption of cell contact did not lead to a difference in the doxorubicin IC_{50} of co-cultured NCI-H929 plasma cells harvested from either control or GD osteoclast cultures. Plasma cell purity remained high (mean >94%) with no difference in purity between plasma cells harvested from either GD or control co-culture. However, the increase in melphalan IC_{50} observed in GD co-culture could be secondary to a small contaminating population of monolayer cells that are more resistant to melphalan or metabolically active than the equivalent number of admixed control monolayer cells. This would lead to an erroneously high MTT absorbance

reading. Although, it could be argued that a small contaminating population of adherent cells did not lead to a higher doxorubicin IC_{50} in the contact co-culture of NCI-H929 cells with either control or GD derived monolayers. However, drug assays of GD and control adherent mononuclear cultures were performed to exclude this possibility.

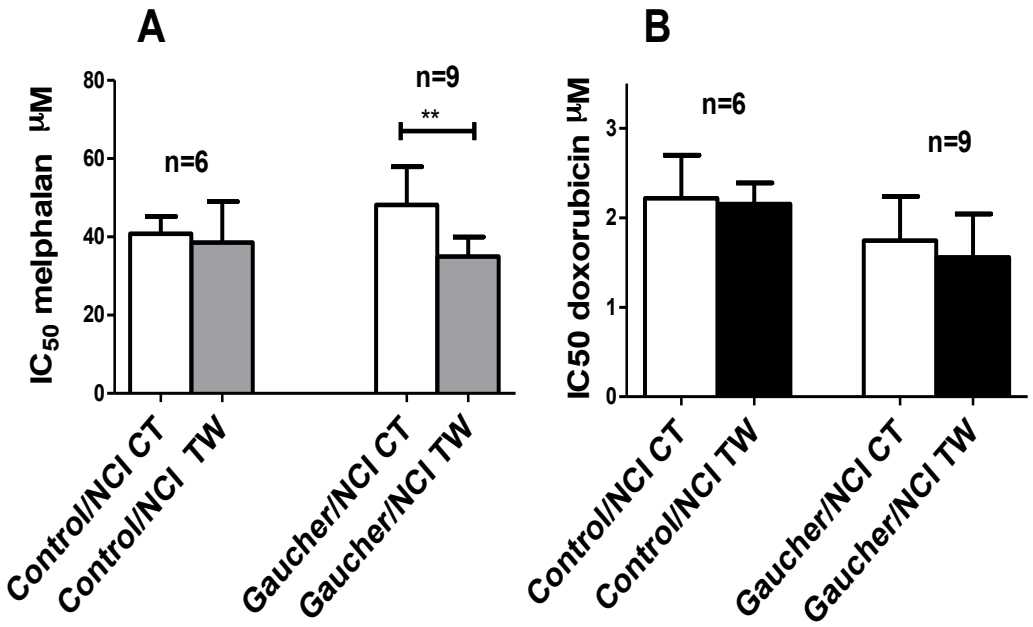


Figure 4-22. (A) Melphalan or (B) Doxorubicin IC_{50} of co-cultured NCI plasma cells (D14-21) in paired experiments utilising trans-well inserts.
CT (Contact), TW(Trans-well). Mean+SD plotted. **p<0.01 (paired t-test)

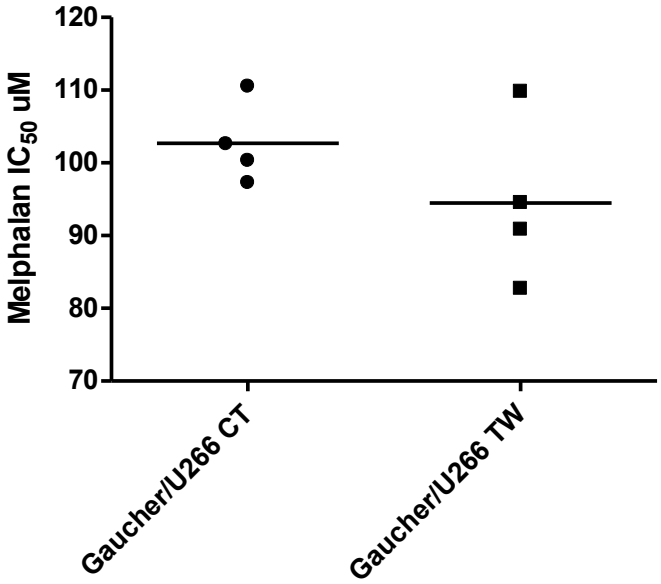


Figure 4-23. Melphalan IC_{50} of GD co-cultured U266 plasma cells (D14-21) in paired experiments utilising trans-well inserts.
CT (Contact), TW(Trans-well). Mean indicated by line.

4.3.4.2 DRUG SENSITIVITY OF MACROPHAGE CULTURES

Control or GD monocytes ($4x10^4$ cells) were plated, in triplicate, in 96-well plates and grown in OC medium for 18 days. Adherent cells were exposed to doubling concentrations of melphalan and doxorubicin for 48 hours before being performing a MTS dye reduction assay (Figure 4-24). Drug sensitivity of Gaucher and control adherent monolayers was reported as a ratio to the corresponding untreated well.

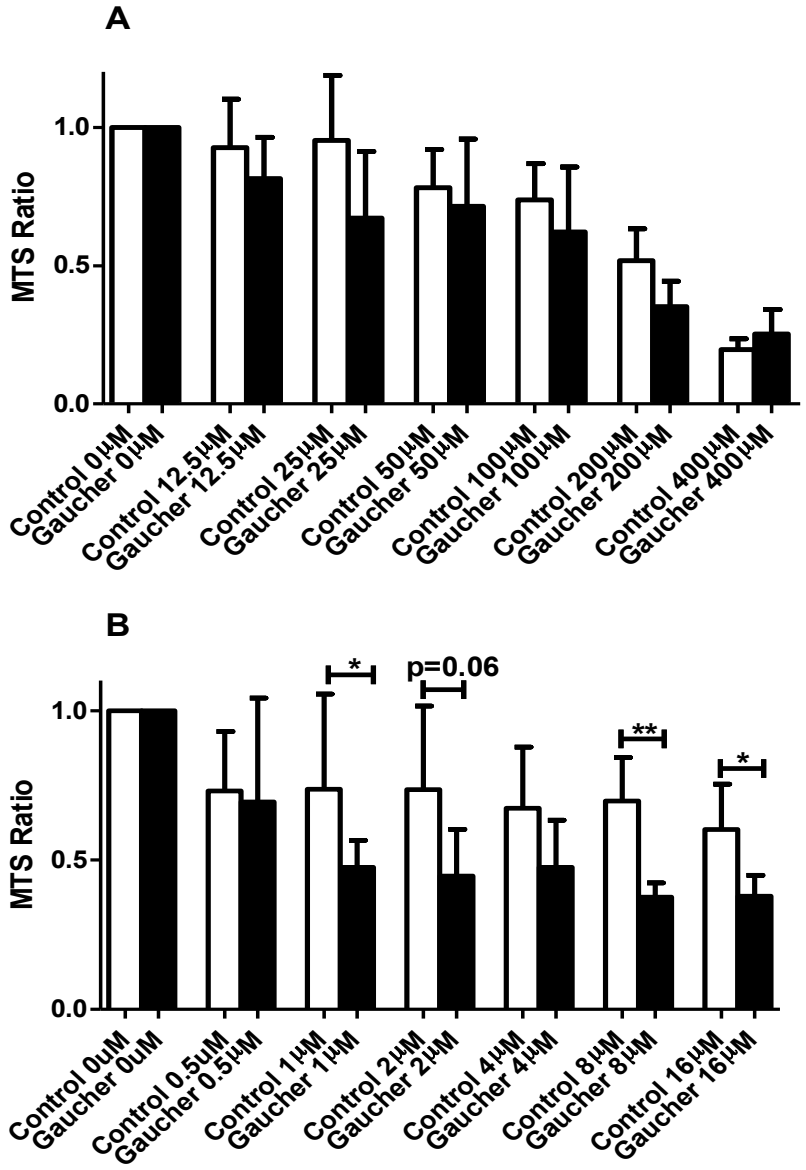


Figure 4-24. Drug sensitivity of day 20 osteoclast cultures following 48 hour exposure to (A) melphalan or (B) doxorubicin as determined by MTS dye reduction assay. Control (n=5), Gaucher (n=5). Mean+SD. *p<0.05 **p<0.01

There was no difference in melphalan sensitivity of cultured Gaucher monocytes compared to control monocytes. GD derived monolayer cells were statistically more sensitive to doxorubicin at several concentrations (1μM, 8 μM and 16 μM) compared to control monolayers (Figure 4-24). Therefore it can be concluded that the small contaminating population of GD monolayer cells is not the explanation for the elevation in melphalan IC_{50} of harvested NCI-H929 plasma cells. Despite an equal number of contaminating control monolayer cells in co-culture, elevation in the melphalan IC_{50} was not demonstrated for either the U266 or NCI-H929 cell lines.

4.3.4.3 PARP-CLEAVAGE

The MTT dye reduction assay was used to calculate the IC_{50} of chemotherapeutic drugs in plasma cells harvested from co-culture, as detailed above. The principle of this test is the mitochondrial reduction of MTT reagent to a purple foramen which is detectable by colorimetric assay. This assay is commonly used as a measure of cell proliferation, drug toxicity or both. However, limitations of the MTT dye reduction test include large variations in absorbance due to alterations in metabolic activity.

PARP is an 116kDa intra-cellular protein that is cleaved into an 85kDa fragment during the terminal stages of apoptosis. In order to confirm that GD monolayers protected plasma cells from apoptosis, western blotting for PARP cleavage of co-cultured (D14-21) NCI-H929 plasma cells harvested from GD or control monolayers was performed. Harvested NCI-H929 cells were re-suspended in culture medium and incubated for 2 hours on tissue culture plastic to increase plasma purity by adhering out contaminating macrophages. Plasma cell purity remained high from both control (99±0.5%; n=6) and GD co-culture (97±1.4%; n=5). Harvested NCI-H929 plasma cells were then exposed to 50μM melphalan for 24 hours before cell lysates were made. Optical densitometry was used to determine band intensity and the ratio of cleaved PARP:total PARP was calculated (Figure 4-25). PARP cleavage was significantly higher in controls compared to GD patients ($p<0.01$) implying a protective effect of GD monolayers secondary to decreased activity of intracellular caspases. Lysates of stock NCI-H929 plasma cells (S) were included as negative controls.

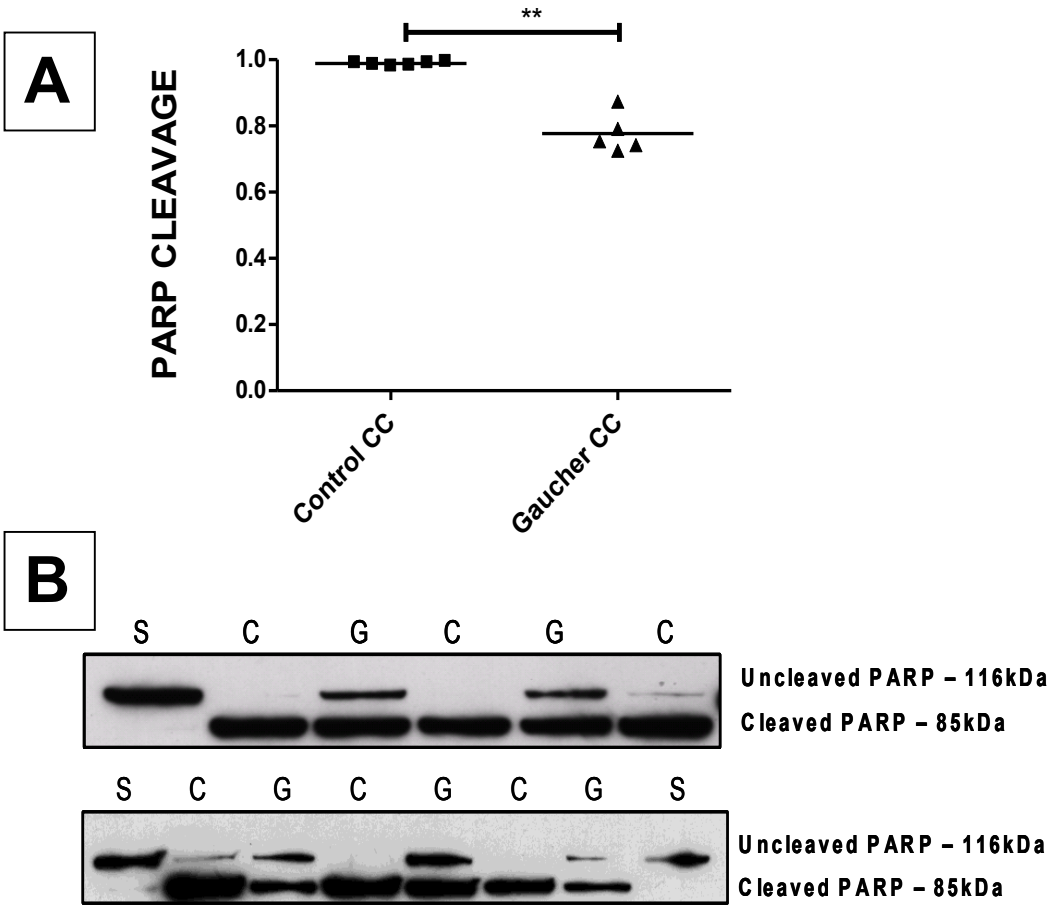


Figure 4-25. (A) PARP cleavage as determined by optical density. (B) Western blot of melphalan treated NCI-H929 cells following co-culture (C=Control, G=Gaucher) compared to untreated stock NCI-H929 cells (S).

4.3.4.4 PLASMA CELL PRIMING

GD monolayers prime plasma cells for survival against melphalan as demonstrated by an elevation in drug IC_{50} and decreased PARP cleavage. It was, therefore, hypothesised that this survival advantage was due to the ability of GD monolayers to alter the basal levels of pro and anti-apoptotic proteins in co-cultured plasma cells. The experiments detailed below were performed using NCI-H929 cells.

4.3.4.5 *TP53* EXPRESSION

p53 is a tumour suppressor protein and many different cellular stressors, including chemotherapy, can induce expression. Induction of *p53* has several different affects, including the up-regulation of DNA repair mechanisms, cell-cycle arrest and initiation of apoptosis in unsalvageable cells. Western blotting for *p53* content was performed in lysates of stock NCI-H929 cells either untreated or following 24 hours exposure to 25μM and 50μM melphalan (Figure 4-26). Lysates of drug naive or chlorambucil treated primary chronic lymphocytic leukaemia cells (CLL) were used in control lanes. NCI-H929 plasma cells had constitutionally high expression of *p53* and this was not increased further by melphalan. The *p53* pathway in NCI-H929 cells is thus dysfunctional, as in many other immortalised cell lines. Expression of *p53* in the CLL cells increased with escalating chlorambucil concentration (Lanes D, E and F).

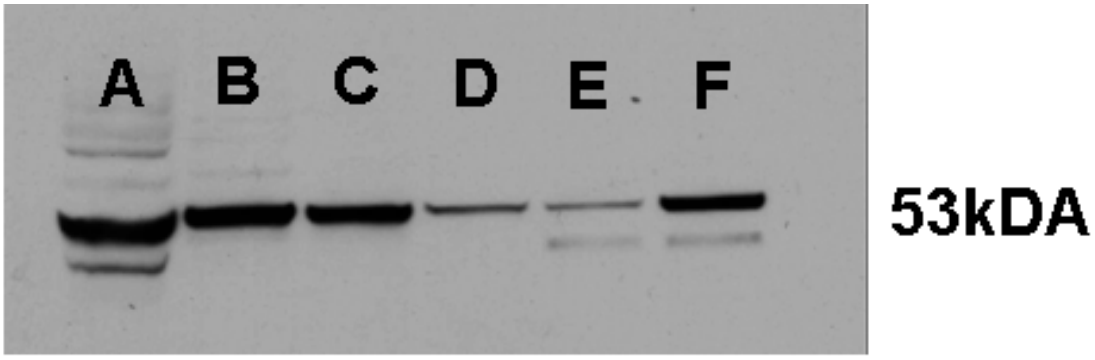


Figure 4-26. p53 expression in NCI-H929 cells as determined by western blotting (30μg protein/lane).
Lanes (A) untreated NCI (B) 25μM melphalan NCI (C) 50μM melphalan NCI (D) untreated CLL cells (E) 20μM chlorambucil CLL cells (F) 50μM chlorambucil CLL cells.

4.3.4.6 INTRINSIC APOPTOSIS PATHWAY - PROTEINS

Lysates were made from untreated NCI-H929 cells following co-culture (D14-21) with control (n=6) or GD monolayers (n=6). Mean plasma cell purity, determined by Giemsa staining, remained greater than 97%, irrespective of monolayer origin. The basal levels of regulatory proteins within the intrinsic death pathway were investigated. This included two pro-apoptotic proteins (Bim and PUMA) and three anti-apoptotic proteins (Bcl-2, Bcl-xL and Mcl-1), see Figure 4-27. In this set of

experiments, stock lysates (S) were made from NCI cells grown in R10 medium whereas co-cultured plasma cells were incubated in OC medium.

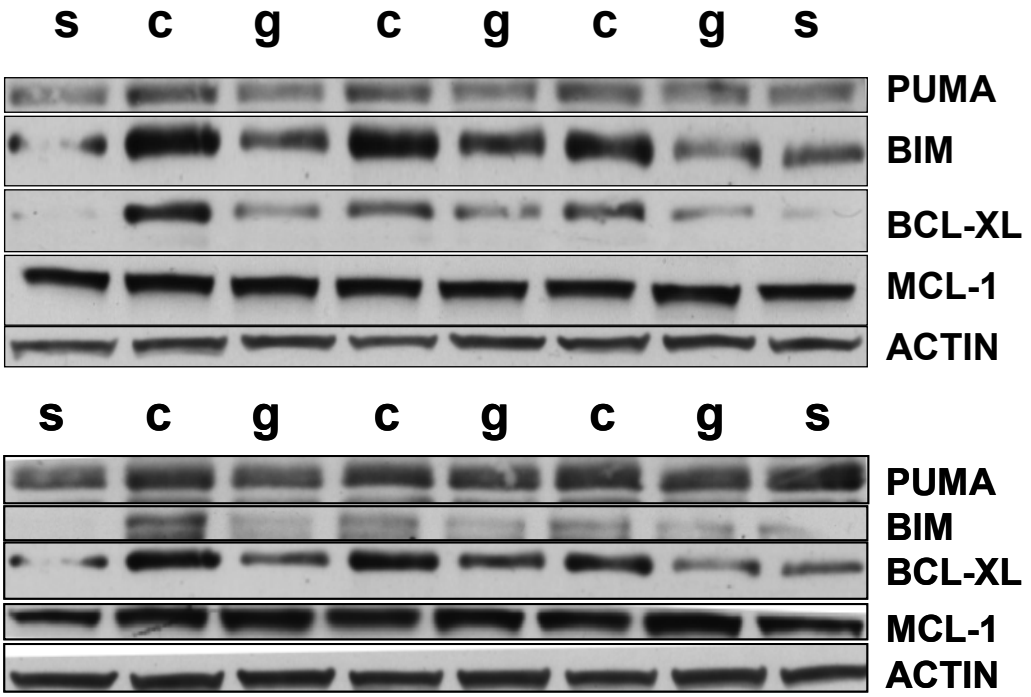


Figure 4-27. Western blotting for pro and anti-apoptotic proteins from untreated co-cultured NCI-H929 plasma cells (D14-21) compared to stock NCI-H929 plasma cells.
Gaucher ([G], n=6), control ([C], n=6) and stock cells ([S], n=4).

The optical density of proteins was quantified by scanning densitometry. Compared to GD co-culture, NCI-H929 cells harvested from control co-culture demonstrated increased basal levels of the pro-apoptotic protein Bim ($p<0.05$) and the anti-apoptotic protein Bcl-xL ($p<0.05$) (Figure 4-28). In addition PUMA was up-regulated in the majority of control co-cultures although, based on optical density, statistical significance was not reached ($p=0.09$). Mcl-1 was present at equivalent levels irrespective of the monolayer origin. Bcl-xL, Bim and PUMA were expressed in equivalent amounts in NCI cells harvested from GD co-cultures compared to stock NCI cells (n=4). Bcl-2 was not expressed in the NCI-H929 cell line. Integrity of the antibody was confirmed subsequently by the detection of Bcl-2 in primary CLL cells.

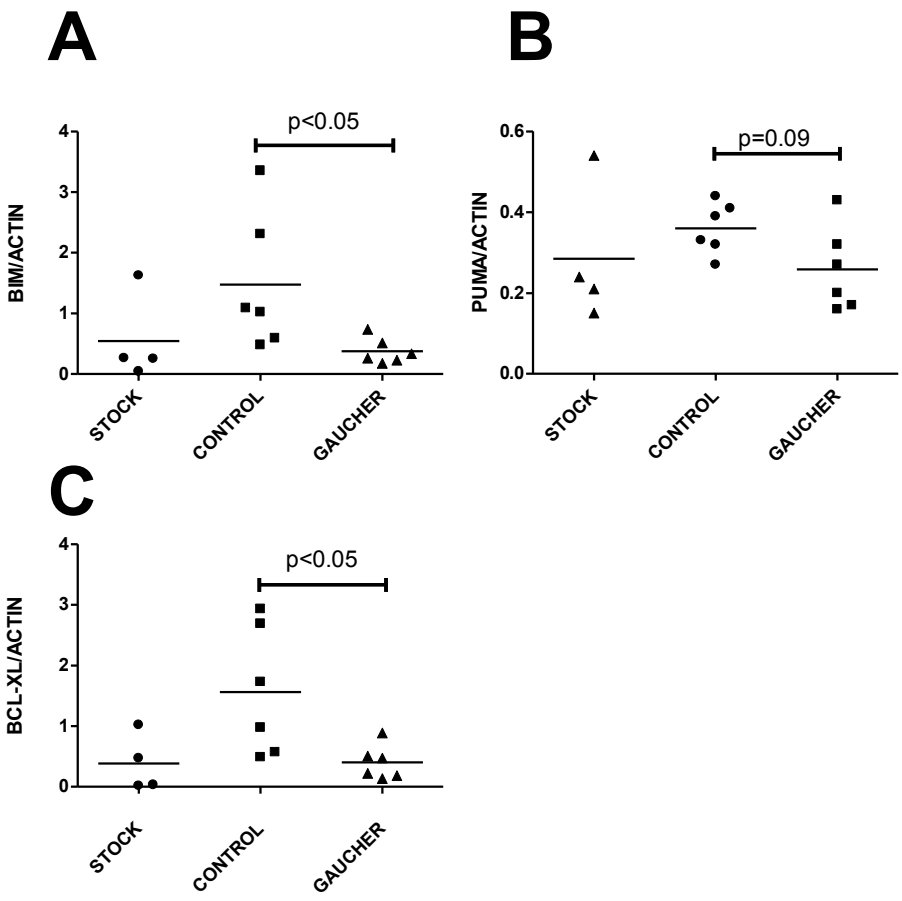


Figure 4-28. Optical density of Bim, PUMA and Bcl-xL of NCI-H929 cells harvested from co-culture (D14-21) compared to lysates of stock NCI cells. Mean represented by plotted line.

The intrinsic, mitochondrial apoptotic pathway is complex and involves a rheostat of regulatory proteins. Activation of cell surface tyrosine kinases including the epidermal growth factor receptor, leads to the phosphorylation of ERK, and the subsequent down-regulation of Bim expression. NCI-H929 cells harvested from either control or GD monolayers had equivalent levels of both ERK and phosphorylated ERK. Therefore BIM is up-regulated in NCI-H929 cells harvested from control co-culture by an ERK-independent pathway.

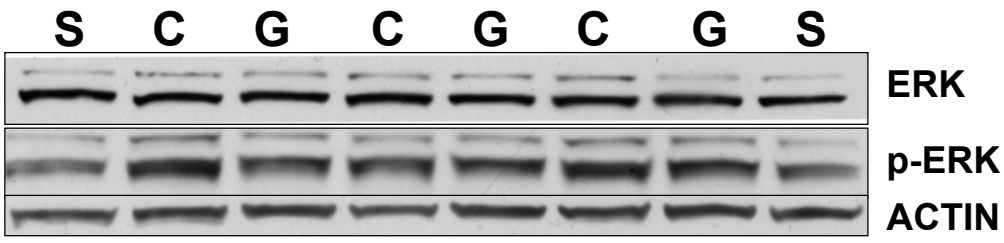


Figure 4-29. ERK and p-ERK expression of harvested NCI cells from GD (G) or control (C) co-culture compared to stock cells (S).

4.3.5 EFFECT OF CO-CULTURE ON OSTEOCLAST NUMBER

The results reported above have demonstrated that GD patients generate more D21 TRAP+ve cells in culture than controls when monolayers are grown alone in OC medium. U266 ($7.5x10^4$) and NCI-H929 ($3 x10^4$) plasma cells were co-cultured with D14 osteoclast cultures from GD patients or controls before termination on D21. Coverslips were fixed, stained for TRAP and the number of osteoclasts was determined (average of 2 coverslips) for comparison to that generated in paired osteoclast only wells. All co-culture wells exhibited a decrease in adherent monolayer cell density on recovered cover-slips in comparison to non co-culture wells. Additionally, co-culture with the U266 cell line led to aggregation and areas of clearing on recovered glass coverslips (Figure 4-16). Despite this, Gaucher co-culture led to an increase in the number of osteoclasts generated for both the U266 ($p<0.05$; Wilcoxon signed rank test) and the NCI-H929 ($p<0.05$) myeloma cell lines. In comparison, control co-culture did not lead to an increase in osteoclastogenesis and the number of osteoclasts generated remained low (Figure 4-30; Figure 4-31). GD co-culture led not only to an increase in osteoclast numbers but, subjectively, to larger multi-nucleate TRAP+ve cells compared to either culture alone or co-culture. Many TRAP+ve cells extended cytoplasmic processes to neighbouring osteoclasts or pre-osteoclasts within the immediate vicinity.

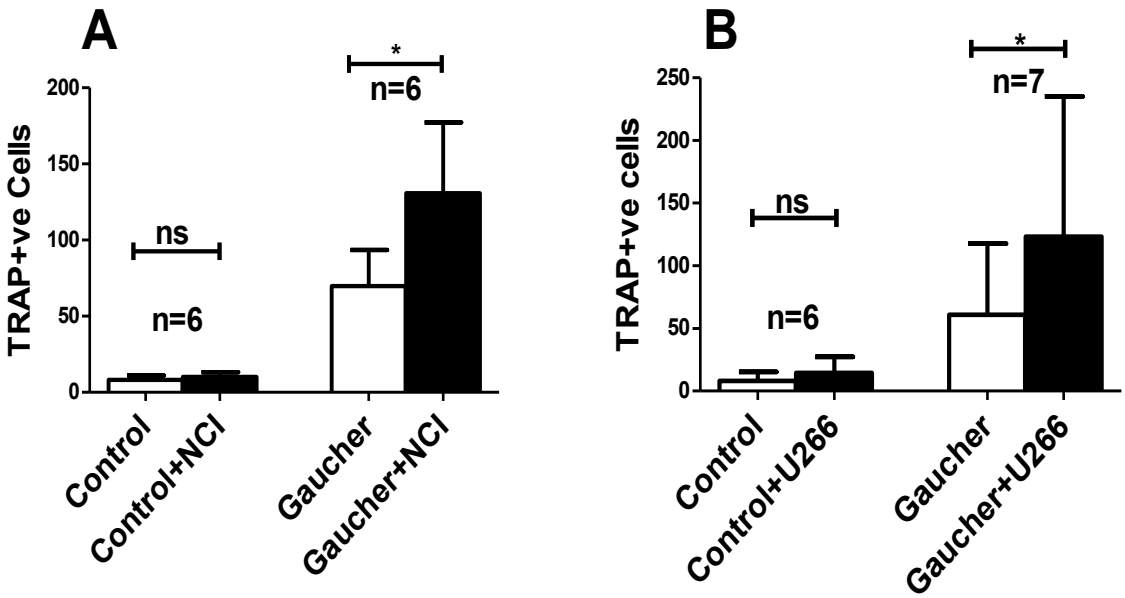


Figure 4-30. Number of TRAP+ve cells generated in contact co-culture (D14-D21) for the (A) NCI-H929 and (B) U266 cell lines compared to D21 non co-culture wells.
Mean+SD plotted. *p<0.05

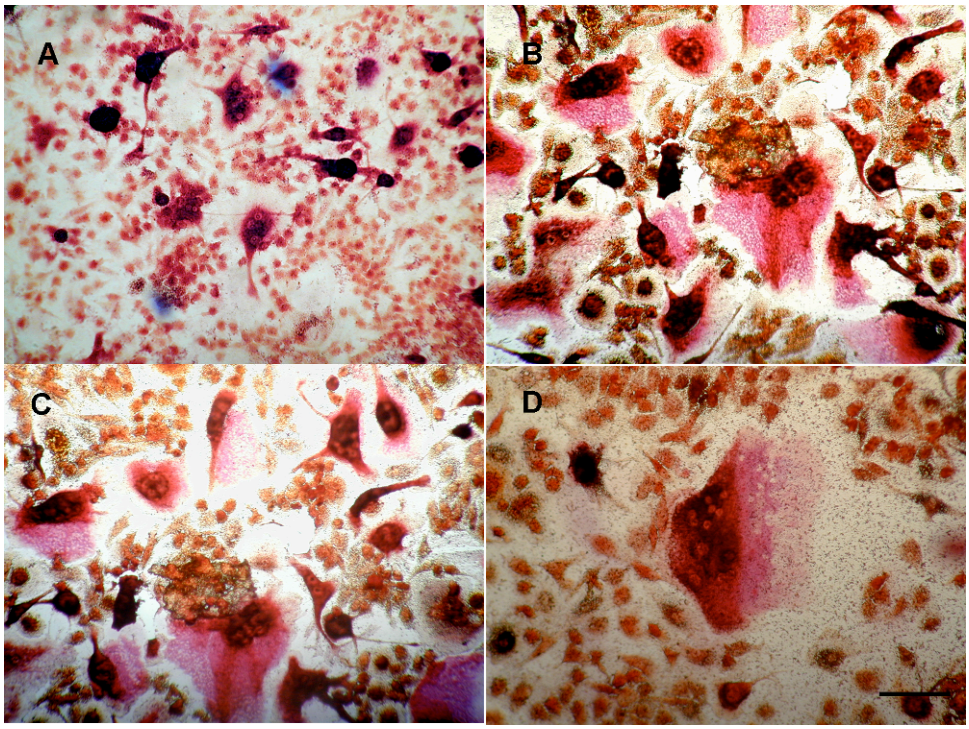


Figure 4-31. Number of TRAP+ve cells post plasma cell co-culture.
TRAP staining in (A) GD osteoclast culture (B) Gaucher NCI-H929 contact co-culture and (C) Gaucher U266 co-culture. (D) Representative image of solitary osteoclast from D21 control culture. Scale bar 100µm.

Co-culture experiments using trans-well inserts were performed to ascertain whether the increase in osteoclastogenesis was contact mediated or secondary to a soluble factor. Paired GD co-culture experiments supported the later as the mean number of osteoclasts generated was similar, irrespective of contact (Figure 4-32).

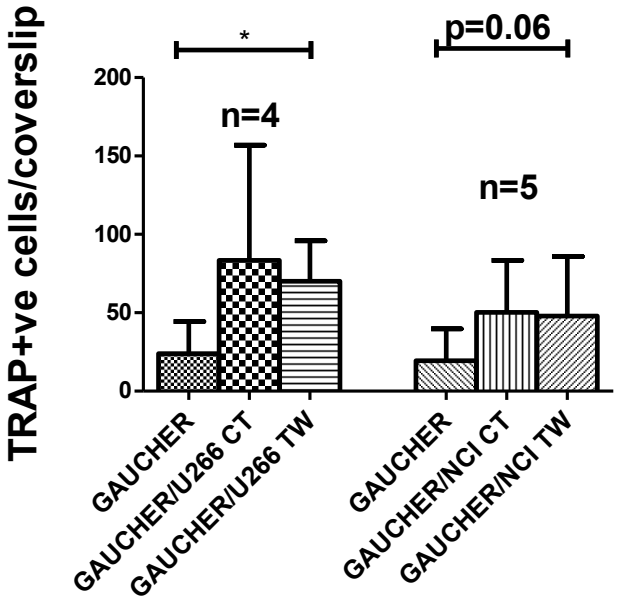


Figure 4-32. Number of osteoclasts generated in contact or trans-well co-cultures compared to sole culture.
Mean+SD plotted. *p<0.05 (Wilcoxon signed rank test).

4.3.6 EFFECT OF ENZYME REPLACEMENT THERAPY ON MELPHALAN IC_{50} AND OSTEOCLASTOGENESIS

MTT dye reduction assay was used to confirm that 1unit/ml of imiglucerase (ERT) was not toxic to PBMCs in culture (data not shown). Imiglucerase 1unit/ml is equivalent to peak blood concentrations achieved in patients post administration and is the standard concentration employed by other investigators in our laboratory.

Paired GD cultures were grown for 21 days +/- the addition of 1ul/ml of imiglucerase. NCI-H929 cells ($4x10^4$) were added on D14 and co-cultured for 1 week (D14-21) as described above (n=4). Plasma cell purity remained high with a minimum purity of 98%. ERT did not lead to a decrease in NCI-H929 cell number in 3 out of 4 experiments and only a reduction of <15% in the other (Figure 4-33). Cell viability (trypan blue exclusion) and the percentage of healthy annexinV-ve/PI-ve NCI-H929 cells were similar between ERT-treated and ERT-naïve wells in 3 out of 4 experiments (Figure 4-33).

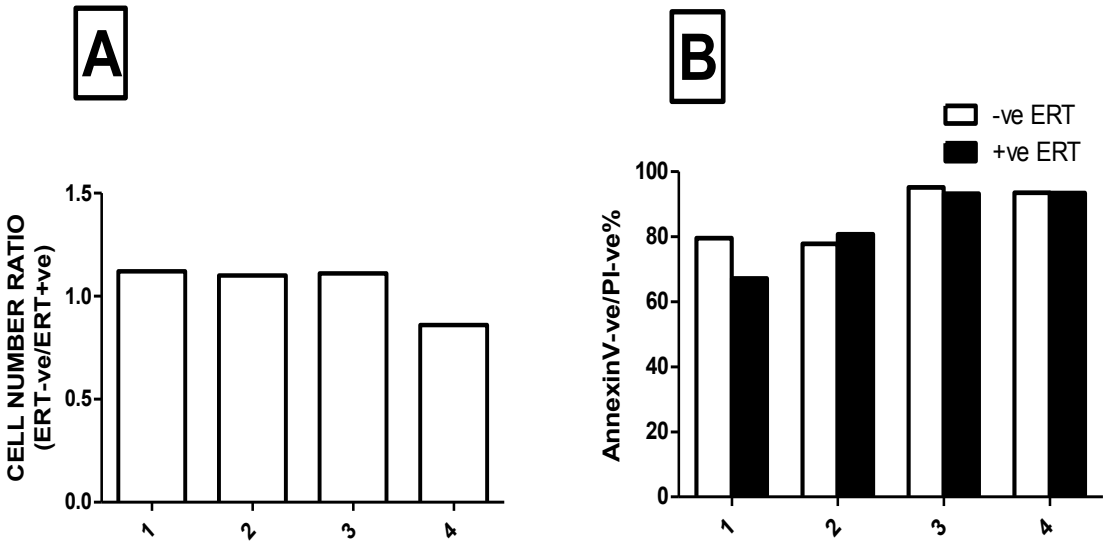


Figure 4-33. GD derived co-culture experiments supplemented with ERT.
(A) Cell enumeration, (B) AnnexinV-ve/PI-ve quantification of NCI-H929 myeloma cells harvested from D21 Gaucher monolayers from 4 different experiments +/- ERT (Patients 1-4).

In addition, ERT-treated wells did not lead to a reduction in the IC_{50} of melphalan as determined by MTT assay 24 hours post-harvest in 3 out of 4 experiments.

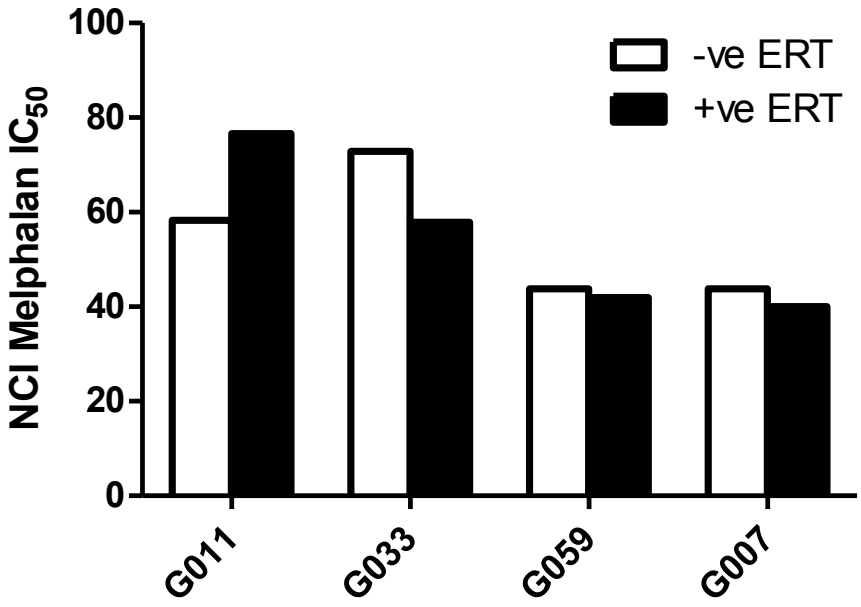


Figure 4-34. Melphalan IC_{50} of co-cultured NCI-H929 harvested from GD co-culture wells (+/- ERT).

However, the number of osteoclasts generated on D21 was reduced in paired ERT-treated wells compared to numbers reported above in control co-cultures. Densities were reduced in all co-culture wells on glass inserts compared to cultures performed in non co-culture. However, ERT seemed to lead to a more pronounced reduction in the monolayer density than ERT-naïve wells, probably due to a lack of large multi-nucleate TRAP+ve cells. Cumulative toxicity of ERT following 3 weeks of culture could not be excluded. Although the number of osteoclasts generated in ERT wells was low, pre-osteoclasts were more evident than that seen in control cultures.

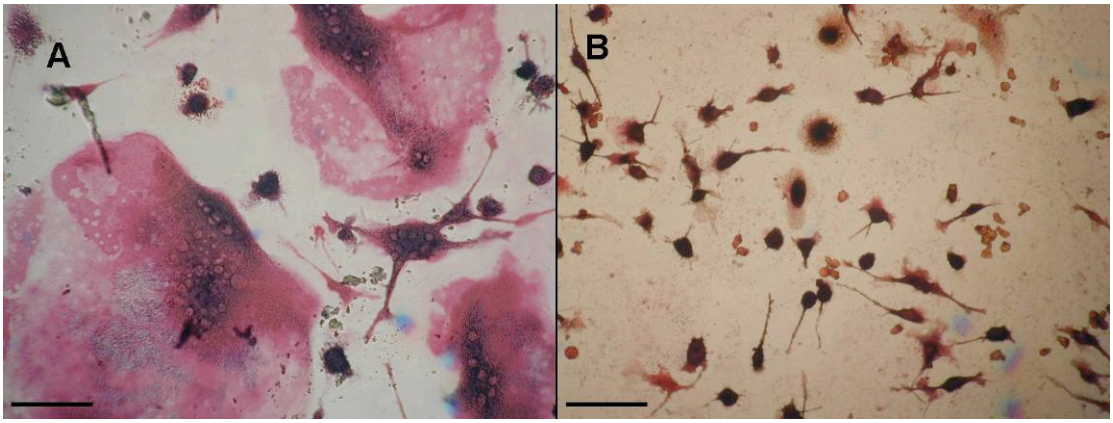


Figure 4-35. D21 TRAP staining of recovered glass inserts following co-culture of NCI-H929 (D14-21) cells in the (A) absence and (B) presence of ERT. Scale bar 100µm.

4.3.7 RESCUE OF DOXORUBICIN-TREATED U266 CELLS

Previously published data have shown that both osteoclast and macrophage cultures derived from peripheral blood are capable of rescuing drug treated plasma cells[245;252]. In addition, the underlying adherent monolayer has been postulated to reduce the proportion of apoptotic tumour cells by phagocytosis. Using our co-culture system, U266 cells were pre-treated with several different concentrations of doxorubicin for 6 hours before being plated alone or on D18 adherent monolayers (control or GD derived) for 72 hours. Cells were harvested on D21 by gentle pipetting, counted and used in annexinV/PI and MTT assays.

Earlier, it was demonstrated that exposure of U266 cells to doxorubicin concentrations ≥1μM for 72 hours resulted in ≥20% annexinV/PI positivity. The 72 hour IC_{50} of doxorubicin in U266 cells was 1.41μM ±0.13 (n=3). Therefore concentrations higher and lower than the IC_{50} were used to establish whether Gaucher monolayers could rescue doxorubicin-treated plasma cells from apoptosis more efficiently than those derived from controls. As described above doxorubicin concentrations of 0.5μM or above led to a near absence of proliferating cells at 72 hours.

4.3.7.1 PLASMA CELL NUMBER

Plasma cell purity was determined morphologically following Giemsa staining. Mean plasma cell purity remained greater than 90% from all co-culture wells irrespective of the origin of the monolayer (GD or control).

Significantly higher numbers of plasma cells were harvested from U266-alone wells compared to control culture following 72 hour exposure to 0μM ($p<0.01$; Wilcoxon signed rank test), 0.5μM ($p=0.06$), 1μM ($p<0.05$), 2μM ($p<0.01$) or 4μM ($p<0.01$) of doxorubicin. There was no difference in the number of plasma cells harvested from GD co-culture compared to control co-culture at all tested concentrations.

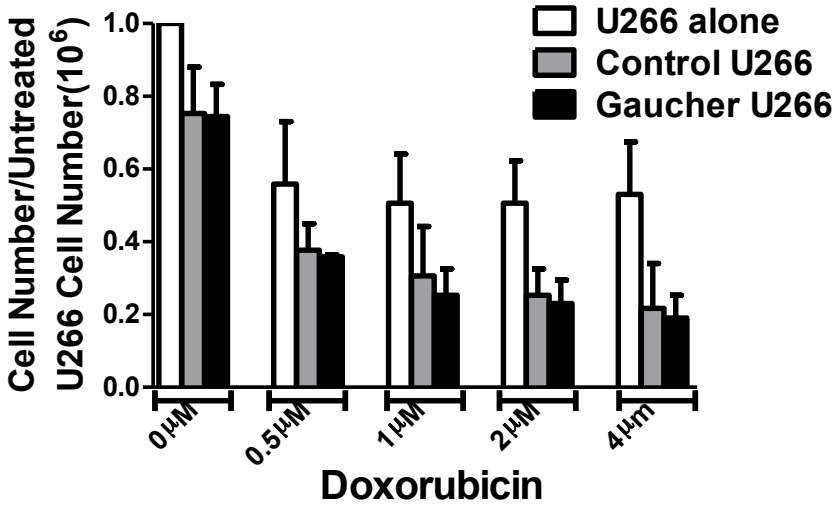


Figure 4-36. Enumeration of doxorubicin pre-treated U266 cells recovered from culture. Recovered U266 cells were counted and compared to the number obtained from the untreated U266-alone well. All datasets represent mean values of greater than n=8, accept for U266 cells pre-treated with 0.5μM of doxorubicin where n≥3 is represented (Figure 4-36). Data represents Mean+SD.

4.3.7.2 ANNEXIN V/PI QUANTIFICATION

Control co-culture wells had a lower number of annexinV+ve/PI+ve cells at all doxorubicin concentrations compared to U266-alone wells. GD co-culture did not lead to a statistically lower number of late apoptotic cells compared to control co-culture. In fact, after exposure to 4μM doxorubicin there was a higher number of annexinV+ve/PI-ve plasma cells in GD co-culture ($p<0.05$) but no difference in the percentage of annexinV+ve/PI+ve cells ($p=0.08$) compared to control co-culture.

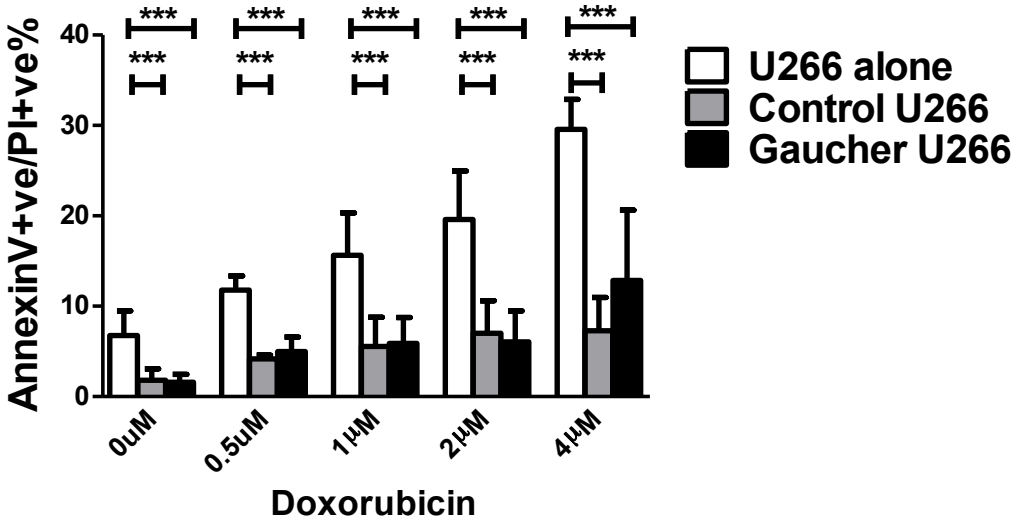


Figure 4-37. Percentage of AnnexinV+ve/PI+ve U266 plasma cells following 72 hours culture alone or co-culture.
Data represents Mean±SD.*p<0.001**

Additionally, the percentage of early apoptotic cells (annexinV+ve/PI-ve) was statistically higher in U266 alone wells than co-culture wells at all concentrations (Figure 4-37; Figure 4-38).

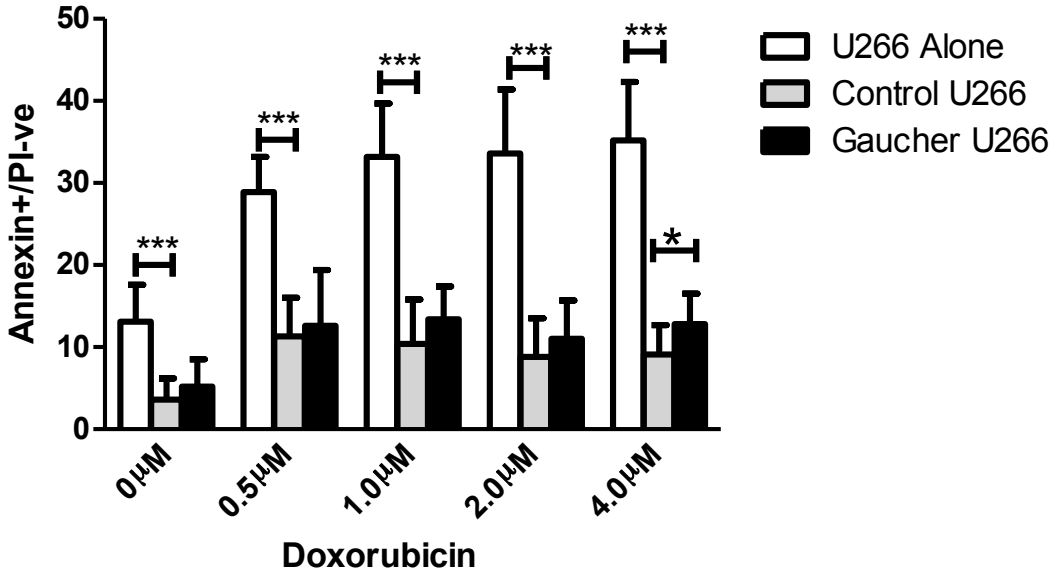


Figure 4-38. Percentage of AnnexinV+ve/PI-ve U266 plasma cells following 72 hours culture alone or co-culture. *p<0.05 ***p<0.001

4.3.7.3 MTT DATA – CELL RESCUE

Doxorubicin treated U266 cells were recovered at 72 hours and a MTT dye reduction assay was performed. U266 cells recovered from either co-culture or culture alone had identical mean values for absorbance in doxorubicin-naïve wells. Surprisingly, mean absorbance increased in plasma cells following exposure to 1μM doxorubicin irrespective of co-culture or origin of the adherent monolayer, possibly reflecting increased metabolic activity of chemotherapy-exposed cells. Control co-culture led to a statistically higher absorbance reading compared to U266-alone wells following exposure to 2μM ($p<0.01$) and 4μM ($p<0.01$) doxorubicin (Figure 4-39). Gaucher co-culture compared to control co-culture led to a lower MTT absorbance value at 4μM doxorubicin ($p<0.05$). This is in keeping with the annexinV/PI flow cytometry data in which control co-culture led to a lower number of early apoptotic cells compared to GD co-culture wells exposed to 4μM doxorubicin.

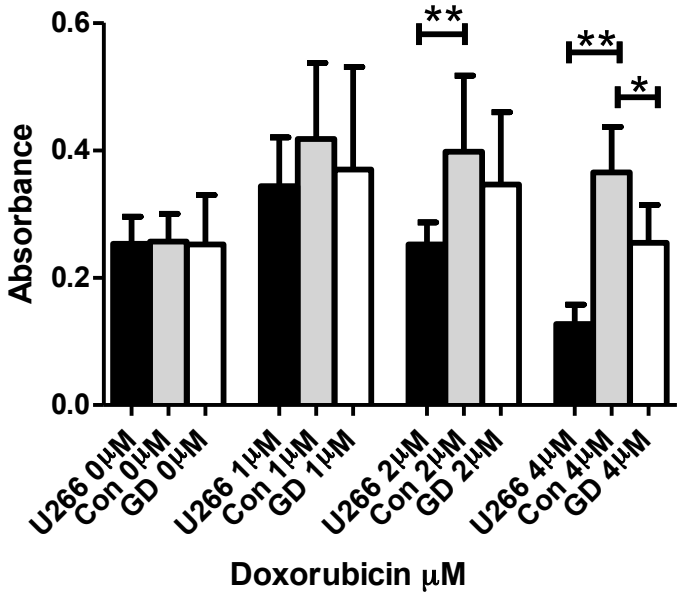


Figure 4-39. Absorbance post MTT assay of U266 cells pre-treated with doxorubicin followed by 72 hours co-culture or culture alone.
Control (Con). Each subgroup consisted of a minimum of n=5. Mean±SD plotted. *p<0.05 **p<0.01

4.3.7.4 HAEMOPHAGOCYTOSIS VERSUS CELL ADHESION

Based on cell number, there were statistically fewer U266 plasma cells harvested from GD or control co-culture wells at all doxorubicin concentrations compared to non co-culture wells. Macrophages are large vacuolated cells that have phagocytic capacity. They ingest pathogens, recycle iron by digesting old red blood cells and destroy apoptosing cells within phagosomes. Macrophages as well as osteoclasts exhibit a cocktail of adhesive proteins.

Co-culture wells were aspirated and washed once gently in PBS prior to removing inserted coverslips and staining with Giemsa. Microscopic examination revealed a significant number of residual clumped plasma cells centred on macrophages from the 0μm and 1μm doxorubicin co-culture wells. In wells exposed to 2μm and 4μm doxorubicin, the number of remaining plasma cells was less. Haemophagocytosis was noted in all co-culture wells but was only seen in the minority of monocytic-derived cells (Figure 4-40; Figure 4-41).

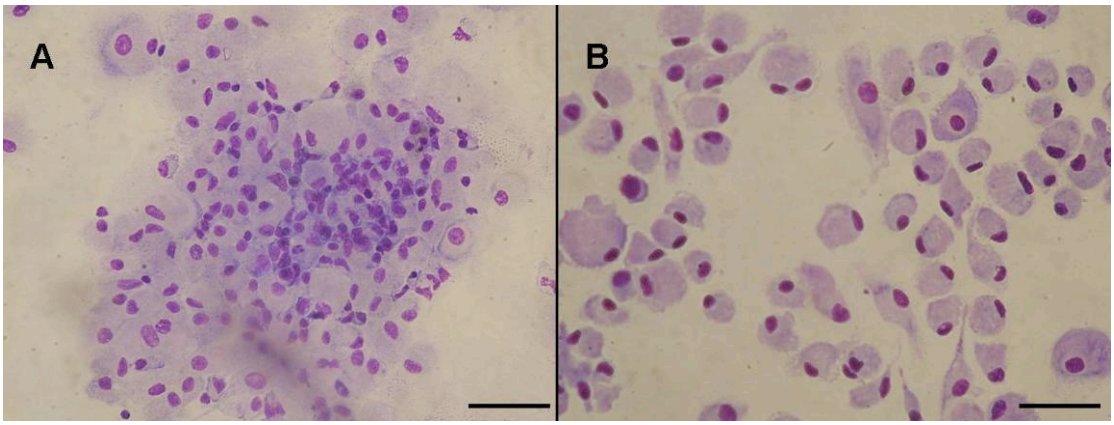


Figure 4-40. Giemsa staining of glass inserts from co-cultured plasma cells following 72 hours culture of myeloma cells treated with (A) 0µM and (B) 4µM of doxorubicin.
Plasma cells are adhered to the underlying monolayer in (A) but are sparse in (B). Scale bar 50µm.

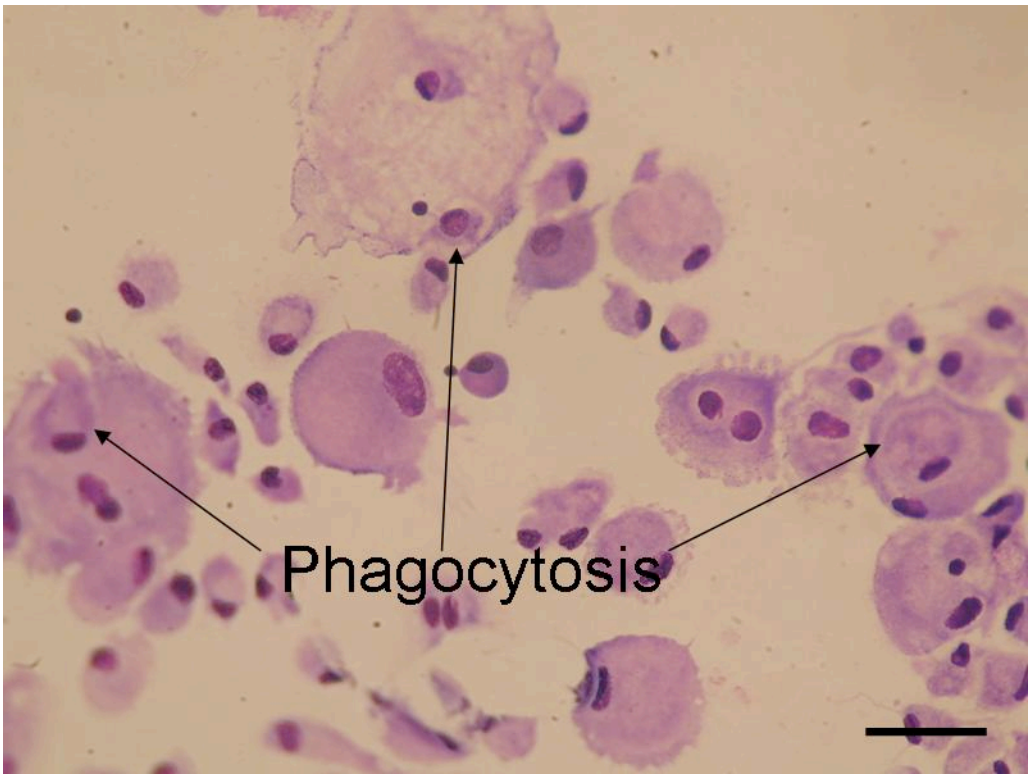


Figure 4-41. Giemsa staining illustrating haemophagocytosis of U266 cells in a co-culture well derived from a Gaucher patient following pre-treated with 4µM doxorubicin. Scale bar 50µm.

A mixture of EDTA/lidocaine was used to remove residual adherent cells from co-culture wells and the number of plasma cells was enumerated by flow cytometry based on side scatter characteristics (Figure 4-42). As demonstrated below, the

forward scatter/side scatter characteristics of plasma cells are different to macrophages. The number of plasma cells harvested decreased with doxorubicin concentration but ranged between 48-75% for untreated wells from 4 individual experiments.

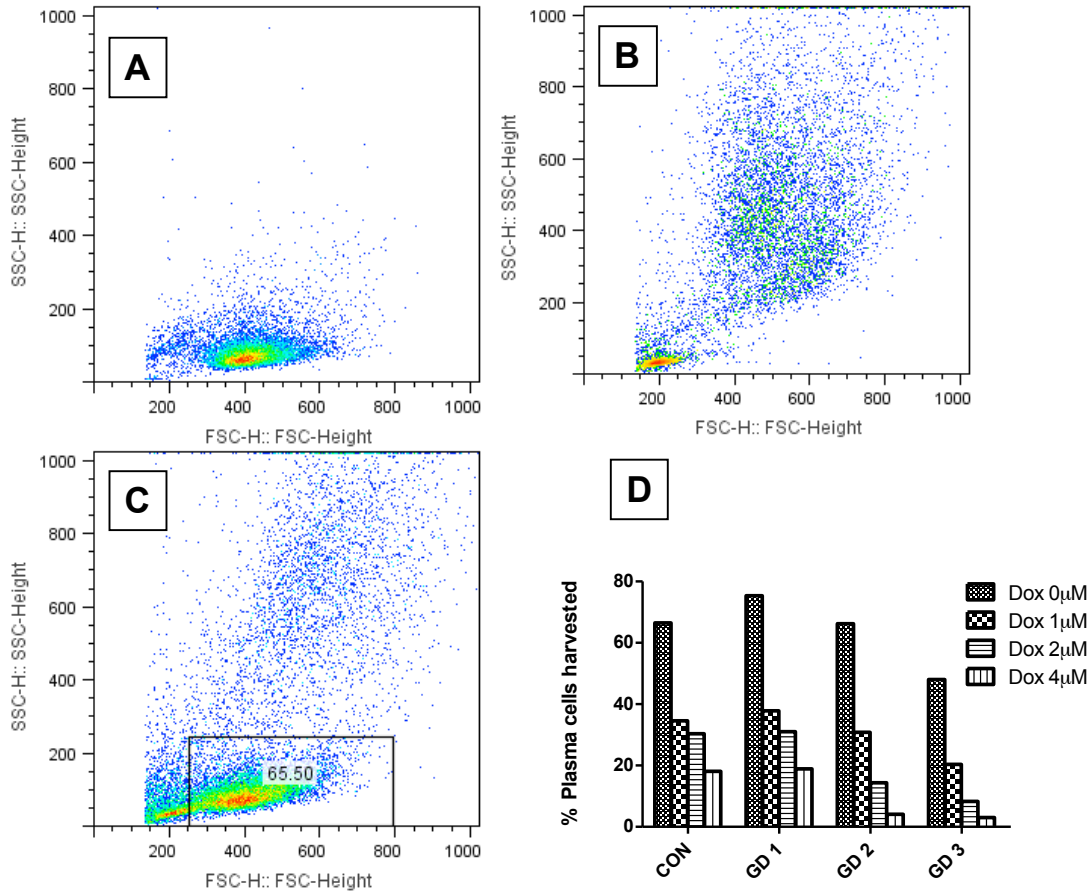


Figure 4-42. Plasma cells are adherent to the underlying monolayer.
Side scatter characteristics from cultures of (A) plasma cells (B) macrophages. Identification of plasma cells based on low side scatter (C) from co-culture wells and (D) the percentage of plasma cells harvested from co-culture wells at the stated doxorubicin concentration (Control, Con; Gaucher, GD).

The number of harvested cells, following exposure to lidocaine/EDTA, was counted using a haemocytometer and the number of adherent myeloma cells was calculated by multiplying by the percentage of plasma cells determined by flow cytometry (Figure 4-43). Based on these data, plasma cell adhesion does not explain the numerical deficit between plasma cells aspirated following co-culture compared to culture alone. For all co-culture wells (GD or control), the number of cells harvested compared to culture-alone was at least $2x10^5$ lower. A combination of haemophagocytosis and cell adhesion may be responsible for this observation.

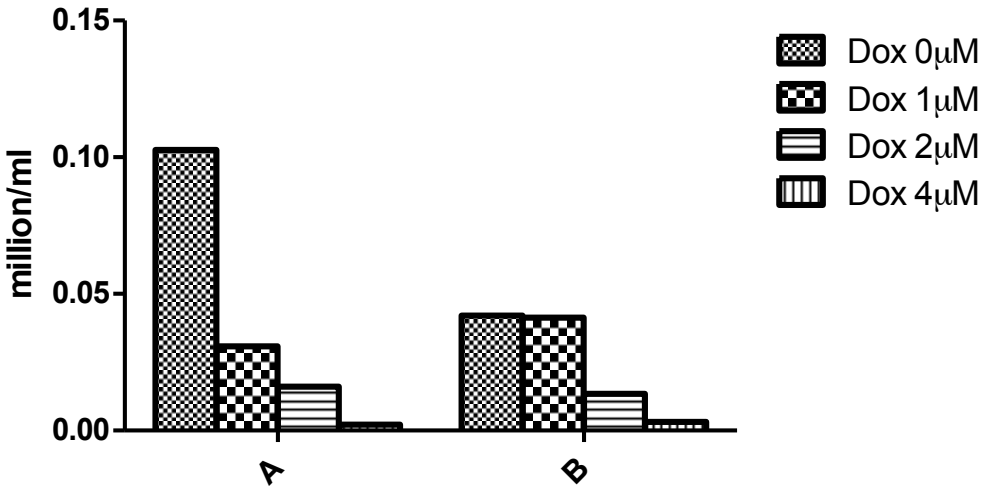


Figure 4-43. Absolute number of plasma cells identified from harvested co-culture wells based on total cell number and plasma cell % as determined by flow cytometry from two experiments.

It was then hypothesised that adherent plasma cells were less healthy than the non-adherent fraction. To-Pro-3-iodide was used to identify the apoptotic component (low side scatter) for both the non-adherent and adherent plasma cells fraction. At all doxorubicin concentrations, the apoptotic concentration was higher for the adherent fraction (Figure 4-44). The percentage of annexinV+/PI+ve cells remained constant in the non-adherent fraction irrespective of doxorubicin concentration (1-4μM). In contrast, the percentage of TO-PRO+ve apoptotic cells in the non-adherent fraction increased with increasing drug concentration (Figure 4-45).

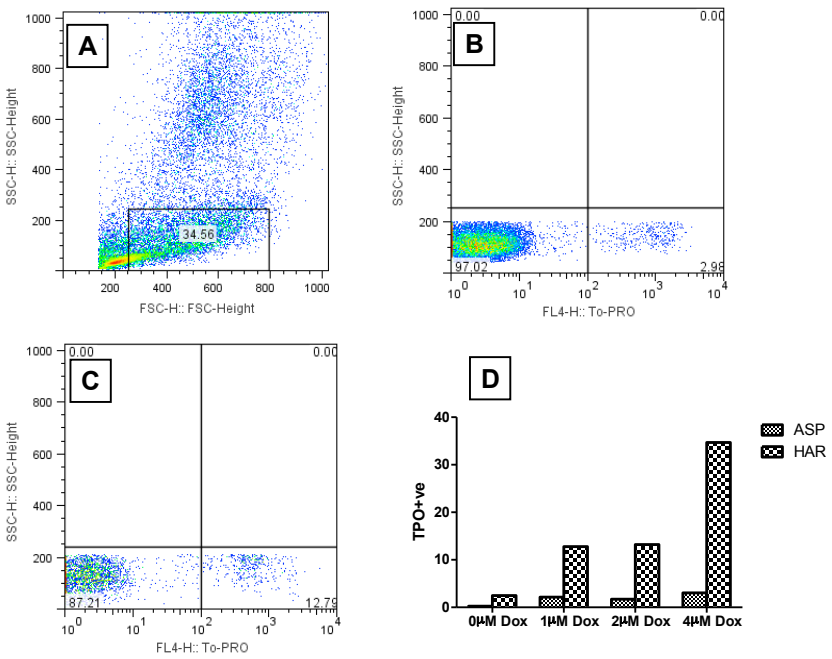


Figure 4-44. Comparison of the viability of co-culture U266 plasma cells pre-treated with doxorubicin (adherent versus non-adherent).
Plasma cells identified on side scatter (A) were subjected to staining with To-Pro; (D) enumeration of the percentage of apoptotic cells from the non-adherent (ASP) and adherent plasma cell populations (HARV), n=1. Illustrated is the number of apoptotic plasma cells from the non-adherent (B) and adherent (C) populations 3 days after a 6 hour pulse of doxorubicin.

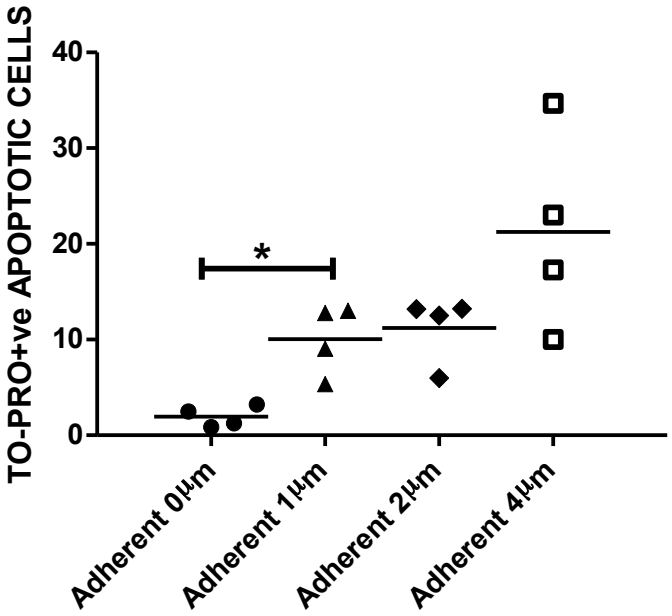


Figure 4-45. Graph showing the percentage of adherent TO-PRO+ve plasma cells (derived from combined Gaucher/Control data) recovered from the co-culture of doxorubicin treated U266 cells (n=4). Mean represented by plotted line.

4.4 DISCUSSION

These results, demonstrate, for the first time potential differences in the bone marrow microenvironment that may render malignant plasma cell in GD patients more resistant to chemotherapy. However, neither a proliferative nor a survival advantage of a GD monolayer on co-cultured myeloma cell lines was demonstrated when compared to a control monolayer. Peripheral blood monocytes from GD patients generated more osteoclasts than control monocytes at D21 when cultured alone or in the presence of plasma cells.

Flow cytometry confirmed monocytes from GD patients to have less glucocerebrosidase activity than those derived from controls (Chapter 3). Kinetic studies have shown that monocytes, relatively young haematopoietic cells, have a short half life in peripheral blood (measurable in hours)[426], before migration and differentiation into tissue macrophages. Organ macrophages, on the other hand, may have a life span measurable in years[391]. Therefore, our 21 day culture experiments, may be of insufficient time to allow GD monocytes to either accumulate significant quantities of glucosylceramide or to develop pathological levels of lysosomal dysfunction. Experiments using CBE, an inhibitor of lysosomal glucocerebrosidase, have demonstrated the accumulation of glucosylceramide at 72 hours in the THP1 cell line and by D15 in fibroblast cultures, as determined by thin layer chromatography[427]. In contrast, glucosylceramide accumulation was only seen in the liver of the L444P mouse model after 7 months by electron microscopy, but not before[330]. However, gammopathy, follicular hyperplasia and plasmacytosis were noted in 2 month old L444P mice, despite an absence in accumulation of glucosylceramide (thin layer chromatography) in the liver, spleen and brain, although the bone marrow was not examined. Therefore glucosylceramide accumulation is not a necessity for gammopathy. In addition, as reported here, abnormalities of lipid trafficking and lysosomal dysfunction are evident in the monocytes of GD patients.

GD derived mononuclear cell cultures generated more TRAP+ve cells compared to controls at D21 in the presence of RANK-L and M-CSF. This is a repeat observation of prior work from our laboratory, awaiting publication. There was significant heterogeneity in the number of D21 osteoclasts generated between individual GD

patients. Speculatively, this maybe due to differences in disease severity, treatment status (ERT or SRT), the timing of blood sampling relative to ERT administration, the concomitant use of bis-phosphonates or differences within baseline glucocerebrosidase activity. GD osteoclasts were larger with higher ploidy than those generated in control cultures. However, individual GD patients tended to produce a similar number of osteoclasts at D21 each time their PBMCs were cultured, suggestive of intrinsic differences between patients in their response to RANK-L/M-CSF. Previously it has been shown that cultured macrophages (D12), generated from the PBMCs of healthy controls, have both a larger cell diameter and higher cytoplasmic:nuclear ratio when incubated with the lysosomal glucocerebrosidase inhibitor CBE[428]. Further work is required to establish inter-individual differences in cytokine receptor density and their ability, on ligand binding, to stimulate intra-cellular pathways of osteoclastogenesis. It has been suggested that homozygous N370S disease is a risk factor for skeletal pathology[52]. Future studies are required to see whether *in vitro* osteoclast production mirrors the severity of bony disease *in vivo*. Currently the literature is sparse regarding osteoclast biology in GD.

Osteoclast cultures derived from controls typically generated fewer TRAP+ve cells compared to GD patients following 3 weeks of culture. In contrast to other groups, who have reported a large number of TRAP+ve osteoclasts by D14, lower concentrations of RANK-L and M-CSF were used in the experiments reported here. This was optimised to create a mixed macrophage/osteoclast model that exploits both the inherent differences in osteoclast generation between controls and GD patients, whilst still reflecting the macrophage-predominant pathology of this disorder. Strategies used to increase osteoclastogenesis by other investigators include, the addition of dexamethasone[259;429], the use of dentine discs (personnel communication, Professor Tim Arnett, University College London), dose escalation of M-CSF or RANK-L[252] and the priming of adherent cells by the non-adherent lymphoid fraction in the first few days of culture[430].

Here, an *in vitro* co-culture system was developed to investigate the effect of peripherally derived mononuclear cells (macrophages/osteoclasts) from GD patients on plasma cell proliferation and survival. This model simplifies the *in vivo* bone marrow microenvironment, which is more complex and includes other components

such as osteoblasts, stromal cells, lymphoid cells, vasculature, extracellular matrix and bone. Plasma cell isolates of high purity (>90%) were recovered from co-culture experiments, which was ascertained morphologically. Other investigators have used CD138 cell surface immuno-staining as a marker of plasma cell purity[431]. However, the data reported here show that CD138 (Sydecan-1) is lost from the cell surface of U266 cells within 72 hours of exposure to doxorubicin and so cannot be used as a marker of purity for cells recovered from co-culture wells. Co-culture experiments using myeloma cell lines have demonstrated a lower proliferation index, a reduction in cell signalling kinase activity, an increased Bcl6:Blimp1 ratio and up-regulation of the STAT3/ERK pathway in the CD138-ve fraction, conferring a phenotype associated with chemo-resistance[425]. Experimental data, including that by Schmidmaier *et al*. (2004), reporting the induction of chemoresistance on co-cultured CD138+ve plasma cells by a stromal layer, neglects the CD138-ve fraction where the more resistance cancer cells may reside[431]. Others have shown a significant loss of CD138 expression from untreated co-cultured myeloma cells lines in the first week of culture [425;432]. The long term co-culture of primary plasma cells (6 weeks) leads to plasmablastic morphology, the loss of mature plasma cell markers such as CD38/CD138 and to the weak expression of more immature markers including CD19 and CD45[303]. Based on the findings of the experiments reported here and previously published data, it was felt that CD138 was not a valid marker of plasma cell purity and could be misleading, therefore plasma cell purity was determined morphologically by Giemsa staining.

In keeping with previous reports, co-culture (D14-21) led to a higher number of NCI-H929 cells (GD or control) compared to myeloma cells grown alone[252], but GD monolayers did not confer an additional numerical advantage. GD patients have been reported to have elevated plasma levels of the B-cell proliferating cytokines, IL-6, IL-8 and IL-10[72;74;78], compared to a healthy control population, although this has not been reported by all investigators. IL-6 is the major growth and survival factor for plasma cells and has been found to be elevated in the blood of non-GD patients with myeloma[433]. Plasma concentrations of IL-6 in GD patients with an M-band have been found to be identical to those without a paraprotein[81]. IL-10 was found to be elevated in nearly all GD patients with a monoclonal band in comparison to only 50% of those without an identifiable M-band[81]. Limitations of these observations

include the fact that normal plasma levels do not necessarily reflect or exclude local elevations within the bone marrow microenvironment. The ability of Gaucher cells to secrete cytokines supportive of plasma cell expansion has been questioned. Boven *et al*. (2004) analysed splenectomy specimens by immunocytochemistry from GD patients and demonstrated that Gaucher cells have the phenotype of alternatively activated macrophages with only weak staining for cytoplasmic IL10 and membrane bound IL-6[58]. These findings suggest that Gaucher cells are unable to produce significant amounts of B-cell stimulating cytokines, however, the ability of neighbouring lymphoid cells within the spleen pulp to secrete these interleukins has not been investigated. Future analysis of culture supernatants within our experimental model may help to document important differences in cytokine profiles between GD and control adherent monolayers.

The number of U266 cells recovered was identical irrespective of co-culture (D14-21). Giemsa staining of recovered inserts demonstrated large numbers of un-recovered U266 cells that had adhered to the underlying monolayer, thus potentially masking a real increase in cell number from co-culture wells. Similar to the U266 cell line, the 5TGM1 myeloma cell line is non-adherent in stock culture, but this cell line was also unable to be fully recovered from co-culture with a stromal layer despite several washes with PBS [434]. Yaccoby *et al*. (2004) showed a lower number of primary myeloma cells recovered from co-culture, possibly due to adherence, although in the face of increased BrdUrd staining and [^{3}H] thymidine incorporation[259]. High purity osteoclast monolayers have been suggested to confer a proliferative advantage over bone marrow stroma in promoting the expansion of myeloma cell lines[252]. However, D21 cultures in our model contained large numbers of macrophages and at present the literature is devoid in documenting their ability to proliferate or adhere to co-cultured plasma cells. In keeping with our findings, Zlei *et al*. (2007) co-cultured U266 cells on bone marrow stroma from healthy controls, the M2-10B4 stromal cell line or PBMC osteoclast cultures, and reported no increase in the number of harvestable cells compared to culture alone[432]. However, others have clearly shown an expansion of U266 cells on primary stromal layers, but to varying degrees[252]. Osteoclast monolayers have been shown to lead to the increased incorporation of BrdU in co-cultured U266 cells[302]. Differences in experimental methodology, including growth medium (the majority of investigators used αMEM),

the timing of initiation or duration of co-culture, cytokine supplementation, composition of the adherent monolayers (stromal cell line, macrophage, osteoclast or primary bone marrow cells) or variation in the proliferating capacity of individual batches of foetal calf serum may add to the uncertainty when comparing results between individual investigators. The same batch of foetal calf serum was used for each experimental protocol reported above.

Both *in vitro* and *in vivo* data support the concept of cell fusion between myeloma cells and osteoclasts, based on cytogenetic analysis, including FISH[311;435]. Co-culture experiments identifying the male-derived U266 cell line by either sex chromosome or BrdU labelling have supported the concept of an osteoclast-myeloma hybrid cell[311]. The authors did not include in their analysis osteoclasts with detectable myeloma cell nuclei based on remnant CD138 immuno-staining (i.e. cells undergoing phagocytosis) but failed to acknowledge that detectable plasma cell nuclei may be pending degradation in the face of an unidentifiable CD138+ve cell membrane. The prolonged culture of U266 plasma cells has been shown to lead to osteoclastic differentiation demonstrated by adherence, TRAP positivity, expression of bone metalloproteinases, RANK-L induced bone destruction and re-arrangement of the actin cytoskeleton[310]. Phagocytosis, adherence or the generation of such hybrid cells would reduce the number of U266 cells harvestable from our co-culture system.

Neither the NCI-H929 nor U266 plasma cells require cytokine supplementation, including IL-6, to facilitate growth or survival in culture. Speculatively, the use of primary plasma cells[259] or the INA-6 or OPC myeloma cell lines[252;436], all heavily reliant on IL-6 for their growth and survival, may have demonstrated a numerical difference between culture alone, control co-culture or GD co-culture. Additionally, reducing the amount of FCS in the OC medium may have made our system more sensitive to any potential differences in cytokine profile secreted by GD or control monolayers. Differences in cytokine production by GD or control monolayers may be of insufficient magnitude to alter the growth cycle of these immortalised cell lines grown in OC medium supplemented with 10% FCS. Abe *et al*. (2004) showed a large difference in OPC cell numbers following 14 days of osteoclast culture compared to culture alone, in medium devoid of IL-6 supplementation; however the expansion of co-cultured U266 cells (IL-6 independent) was dramatically less

impressive[252]. Based on BrdU incorporation, stromal layers have been shown to lead to a vast increase in the percentage of S-phase cells in the IL-6 dependent cell lines, UM-2 and UM-3[437]. Osteoclast monolayers generated from myeloma patients or healthy controls have been suggested, by some investigators, to be equally supportive of primary plasma cell growth[259]. Although others have demonstrated a growth advantage for both IL-6 dependent and independent myeloma cell lines on stromal layers derived from myeloma patients[438]. Bone marrow stromal layers from multiple myeloma patients compared to healthy controls have been shown to up-regulate vascular cell adhesion molecule VCAM-1 expression via a NF-κB-dependent pathway and to secrete more IL-6, secondary to increased signalling via p38 MAPK[438].

Experimental data, reported here, using trans-well inserts, supports a contact dependent increase in NCI-H929 cell number, irrespective of the origin of the monolayer. Co-culture experiments using adhesion assays and neutralising antibodies have shown that very late antigen VLA-4 expressing plasma cells can interact with stromal cells expressing VCAM-1[304]. It has been further demonstrated that the contact co-culture of plasma cells with primary osteoclast monolayers leads to substantially higher levels of IL-6 in the culture medium[252]. Trans-well experiments demonstrated lower IL-6 concentrations than contact co-culture, but were still significantly higher than levels found in the supernatant of osteoclast only cultures[252]. In the experiments presented here, the number of harvested NCI-H929 cells was identical between Gaucher trans-wells and control trans-wells. In summary, based on cell number it has not been possible to show a proliferative advantage of a GD monolayer compared to that derived from healthy controls in contact co-culture for either myeloma cell line or for NCI-H929 plasma cells in a contact-deprived model. Paired experiments led to an identical number of NCI-H929 plasma cells harvested from GD co-culture, irrespective of the addition of ERT. It should be noted that the activity of stored ERT was not confirmed on thawing, adding uncertainty to this conclusion.

Cell cycle analysis demonstrated an equivalent number of U266 and NCI-H929 cells in S-phase on D21, irrespective of whether they were cultured alone or co-cultured with GD or control monolayers. This was somewhat surprising given the higher

mean number of NCI-H929 cells following co-culture. Although there was a mean increase in NCI-H929 plasma cell numbers harvested from co-culture compared to culture alone, many individual experiments did not led to an increase in plasma cell numbers. In addition, by D21 the growth medium from co-culture wells is likely to be relatively more depleted of nutrients, with higher concentrations of toxic by-products of cellular respiration, secondary to the presence of highly metabolic macrophages/osteoclasts. RPMI 1640 contains phenol red and co-culture wells demonstrated a slight change to a darker red colour, indicating lower pH, compared to non co-culture wells by D21, although not dramatically so. Speculatively, cell cycle analysis during the early stages of co-culture, reducing the impact of medium exhaustion and toxin accumulation, may have demonstrated a difference in the percentage of plasma cells in S-phase compared to non co-culture. In summary, GD co-cultures did not confer a proliferative advantage on either cell line compared to control co-culture.

Contact co-culture (D14-21), GD or control, led to a more viable plasma cell population compared to contact-independent co-culture or 1 week culture alone, for both the U266 and NCI-H929 myeloma cell lines, in keeping with prior data[252;259;432;437]. GD co-culture (D14-21), irrespective of contact, did not convey a survival advantage over control monolayers. Cell viability was similar between plasma cell-only wells and those recovered from trans-well inserts, suggesting that soluble factors alone are not sufficient to promote survival. Macrophages are able to digest apoptotic cells and can recognise externalised phosphatidylserine on the cell membrane of damaged cells[264]. There is some evidence that osteoclasts have haemophagocytic activity, based on their ability to digest chondrocytes within the epiphyseal growth plate of rat and mice long bones[439;440]. As discussed above, there are publications supporting the concept of an osteoclast-myeloma fusion cell[311]. Yaccoby *et al*. (2004) reported that cultured osteoclasts have haemophagocytic capacity based on the ingestion of apoptotic plasma cells (trypan blue staining) following exposure to lethal doses of dexamethasone[259]. Although they demonstrated that 95% of their cultures were TRAP+ve osteoclasts, they failed to acknowledge that the remaining 5% of undifferentiated cells were TRAP-ve macrophages with potentially high phagocytic activity. A spectrum of differentiation is likely to exist within the monocytic lineage ranging between non osteolytic TRAP-ve macrophages

and TRAP+ve bone osteoclasts. In support of this concept, histological analysis of peri-prosthetic material, harvested at the time of operative invention, showed that a subset of TRAP+ve/vitronectin receptor+ve cells had both the morphology and CD68 immuno-reactivity of tissue macrophages[441]. A large number of cells within the culture system used in this chapter, were TRAP-ve and thus likely to have significant phagocytic activity.

In these experiments, U266 plasma cells, pre-treated with doxorubicin, demonstrated a concentration-dependent increase in the percentage of TO-PRO+ve cells adhered to the underlying monolayer. In keeping with this finding, Zheng *et al*. (2009), by using antibodies to ICAM-1 to prevent the adhesion of melphalan-treated plasma cells to the underlying adherent monolayer, demonstrated an increase in the percentage of recoverable annexinV positive cells to levels seen in non co-culture wells[245]. The authors concluded that the contact co-culture of macrophages, protected plasma cells from chemotherapy based on annexinV analysis despite their own data suggesting that the apoptotic plasma cell population was adherent and thus not harvestable. The interaction of ICAM-1 with CD11b/18 on alveolar macrophages has been shown, by other investigators, to enhance the phagocytosis of FITC-labelled microspheres[442]. Despite these caveats, it can be concluded that the co-culture of untreated myeloma cell lines (D14-21) with GD or control monolayers leads to a more viable plasma cell population compared to culture alone. Assuming equivalence in phagocytic activity and the ability to adhere apoptotic cells between monolayers, GD co-cultures did not confer an additional survival advantage over control cultures.

Co-cultured NCI-H929 or U266 plasma cells (D14-21) harvested from GD osteoclast cultures, in a contact-dependent manner, led to an increase in the melphalan IC_{50} for both cell lines. This was not seen in plasma cells recovered from control cultures. In addition, NCI-H929 cells harvested from GD co-culture and incubated for 24 hours with 50μM melphalan demonstrated reduced PARP cleavage, compared to those recovered from control co-culture. NCI-H929 cells, primed by GD monolayers, had a statistically higher number of plasma cells in GO/G1 phase of the cell cycle compared to non co-culture cells. Malignant cells arrested in G0/G1 have been shown to be more resistant to chemotherapy[443;444]. Cycling plasma cells in S-phase have lower expression of the anti-apoptotic protein Bcl-2[445], conferring an increased

susceptibility to chemotherapy. The experiments reported here, were designed to establish whether GD monolayers primed plasma cells for survival. In comparison, other investigators, have added drug to the co-culture system, in order to replicate more accurately the *in vivo* model, or studied the rescue of plasma cells pre-treated with chemotherapy[245;252;446].

Bone marrow stromal cells, patient-derived or cell line, have been shown to be sensitive to both melphalan and doxorubicin, two drugs historically used in the treatment of multiple myeloma[447]. D18 osteoclasts/macrophages derived from GD patients were more sensitive at several different concentrations to doxorubicin compared to controls, whereas the MTS ratio following melphalan treatment was equivalent at all tested concentrations. Plasma cell purity was equivalent between wells harvested from control or GD patients following 7 days co-culture (mean purity ≥94%; minimum 90%). These findings exclude a small contaminating population of chemoresistant GD monolayer cells being the explanation for the observed increased in melphalan IC_{50}. Potentially, up to 10% of harvested cells were admixed osteoclasts or macrophages. As GD monolayer cells were comparatively more sensitive to doxorubicin, this may have masked a true increase in the IC_{50} of recovered cells following culture (D14-21). Compared to control co-culture, significance was not reached in doxorubicin treated U266 cells recovered from GD co-culture ($p=0.08$). Additionally, it could be argued that post harvest, during the 24 hour MTT cytotoxicity assay, the plasma cells were still in co-culture, with potentially a plasma cell/monolayer cell ratio of 10:1. Abe *et al*. (2004) added doxorubicin to co-culture wells using a ratio of 20:1 (plasma cells:osteoclast) and reported a protective effect based on cell number only[252]. Yaccoby (2005) claimed that a 1000:1 ratio (plasma cell:osteoclast) was sufficient to prevent dexamethasone-induced apoptosis, based on annexinV analysis only[303]. However, the author did not provide data on recoverable plasma cell number or cell proliferation and, in addition, failed to exclude the adherence or phagocytosis of apoptotic cells to the underlying osteoclast monolayer, thus casting doubt to this observation.

Based on 24 hour MTT drug assay, using doubling concentrations of doxorubicin or melphalan, induction of chemoresistance was not demonstrated in myeloma cell lines primed by control co-cultures (D14-21) compared to culture alone. Prior literature

has reported[252;259] the induction of chemoresistance by bone marrow stroma and osteoclast layers in co-cultured plasma cells, which were in contact. Publications are lacking regarding the effect of macrophages on the chemosensitivity of plasma cells and the report by Zheng *et al*. has several limitations, as discussed above[245]. In contrast, the system used here compares the ability of GD and control monolayers to prime plasma cells for survival. Unlike GD co-culture, control adherent monolayers demonstrated very few TRAP+ve osteoclasts by D21 and consisted almost entirely of macrophages. Whether the difference in osteoclast generation is the explanation for the observed difference in melphalan IC_{50} is speculative. As osteoclasts support myeloma growth and survival, investigators in recent years have studied the impact of novel agents including thalidomide[309], bisphosphonates[252], lenalidomide[308] and bortezomib[307;448] on their ability to impair osteoclastogenesis.

Immortalised myeloma cell lines have an array of pro and anti-apoptotic proteins, which are constitutionally expressed (e.g. Bim, Bcl-xL, Mcl-1 and Bcl-2) and inducible by either cell contact or exposure to chemotherapy[245;449-451]. RPMI-8266 plasma cells, co-cultured on a fibronectin-coated surface, down-regulated Bim expression (pro-apoptotic) and acquired chemoresistance to melphalan[451]. The addition of IL-6, a bone marrow derived cytokine, to cultures of the MDN myeloma cell line led to induction of the anti-apoptotic proteins Bcl-xL and Mcl-1[252;450;452]. In addition, the transfection of U266 cells with a Mcl-1 over-expressing construct has been shown to prevent interferon-induced cell death[453]. Therefore, apoptotic regulatory proteins are inducible and can alter the chemosensitivity of myeloma cell lines. It was hypothesised that GD monolayers by altering the levels of these proteins in co-cultured plasma cells, confer melphalan chemoresistance.

In the co-culture system used here, untreated NCI-H929 cells harvested from D21 control monolayers, had increased levels of the pro-apoptotic protein Bim compared to those from GD co-culture, where expression was low and similar to that found in stock cells grown in R10 without RANK-L/M-CSF supplementation. In the NCI-H929 myeloma cell line, Bcl-2 was not expressed. Curiously, Bcl-xL was also expressed at high levels in untreated NCI-H929 cells primed by control monolayers (D14-21) compared to GD osteoclast cultures. In keeping with these findings, macrophage monolayers matured with the conditioned growth medium of myeloma

cells have been shown to lead to the induction of Bcl-xL expression in co-cultured ARP-1 cells[245]. Bim, in comparison to other pro-apoptotic proteins, has been found to be potent killers of fibroblasts in an *in vitro* cytotoxicity assay[454]. Bim is known to down-regulate the activity of several anti-apoptotic proteins including Bcl-2, Mcl-1, A1, Bcl-xL and Bcl-w[408]. There is a rheostat of inducible pro and anti-apoptotic proteins and in these experiments only a targeted minority have been assessed. It can therefore be speculated that the net induction of pro-apoptotic proteins outweighs the up-regulation of any anti-apoptotic proteins in NCI-H929 cells primed by control monolayers compared to GD co-cultures.

The increase in melphalan IC_{50} for both myeloma cell lines harvested from GD monolayers, compared to control co-culture, was in the order of only 30%. Contact deprivation from the underlying monolayer following harvest, may lead to a rapid loss in plasma cell priming and the subsequent re-balancing of pro and anti-apoptotic proteins. This may have contributed to the failure to demonstrate a protective effect following control co-culture, the inability to induce chemoresistance in harvested cells that were then exposed to doxorubicin and the relatively small increase in melphalan IC_{50} following GD co-culture. Western blot analysis has demonstrated the ability of pro and anti-apoptotic proteins to be induced followed by rapid decline within a few hours of therapeutic challenge[453;455].

In this co-culture system, immortalised cell lines were used due to their constant availability and ability to proliferate to yield high enough cell numbers to perform all outcome measures. It was demonstrated that peripheral lymphocyte subsets of GD patients, including CD19+ve B-cells, have low glucocerebrosidase activity as determined by flow cytometry (see chapters 3 and 5). During the course of this study, no GD patient with a plasma cell disorder underwent bone marrow examination. Clonal plasma cells derived from GD patients, due to low enzyme activity and abnormal sphingolipid metabolism, may have intrinsic differences in their cellular biology, which alter chemosensitivity. Therefore, they may be primed differently to immortalised plasma cells lines generated from individuals without GD. A further criticism of these experiments is that the myeloma cell lines used have intact glucocerebrosidase activity instead of glucocerebrosidase-deficient plasma cells that

may have differences in their cellular biology (discussed below). This should be addressed in future experiments.

Morjani *et al*. (2001) demonstrated a link between multi-drug resistance (MDR) and sub-cellular glucosylceramide accumulation in a breast cell cancer line[456]. Additionally MCF7-resistant breast cancer cells, pre-treated with CBE for 24 hours, demonstrated further increased resistance to doxorubicin. Treatment with glucosylceramide synthase inhibitors had been linked to the increased chemosensitivity of primary and immortalised CLL cells, although the authors failed to demonstrate a reduction in the P-glycoprotein (P-gp) multidrug efflux pump in patient derived samples, suggesting an alternative mechanism[457]. Additionally, their data were based on an MTT assay, which reflects cell proliferation and viability, rather than more specific markers of apoptosis, such as caspase activity or PARP cleavage. Intracellular ceramide has a role in the regulation of cell cycling and the mediation of chemoresistance. Chemosensitivity is linked to the accumulation of ceramide[458]. In contrast, depleted intracellular levels are found in primary tumours, refractory cancers and immortalised malignant cell lines. Ceramide analogues have been shown to induce apoptosis in breast cancer cell lines[458]. GD patients, due to enzyme deficiency, are unable to hydrolyze glucosylceramide to ceramide. Translating this into a clinical setting, there is insufficient literature at present to conclude whether GD patients respond less well to chemotherapy than non-GD patients as both favourable and un-favourable responses have been reported[81;136;161]. There is no consensus on whether myeloma, complicating GD, is an indication for disease modifying therapy[66;161]. As discussed in chapter 2, no patient with an established M-band had escalation in their paraprotein level whilst on ERT. Therefore, it can be argued, as MGUS can progress to myeloma, all patients with evidence of a clonal plasma cells disorder should receive ERT. In this co-culture system, the addition of ERT did not lead to a reduction in the melphalan IC_{50} of co-cultured NCI-H929 plasma cells. However, it should be noted, that ERT was obtained and stored frozen from the remnants of patient vials. A major limitation to the data presented above is that the activity of stored ERT was not confirmed on thawing. This should be addressed in future experiments in order to validate this assay and the reported findings.

Autophagy is a lysosomal dependent process involved in the catabolism of cellular organelles and waste products. It has been speculated to play an important role in the modulation of chemosensitivity[459]. In a mouse model of neuronopathic GD, impaired autophagy has been demonstrated in neurons and astrocytes[460]. It has been shown that mice haplo-deficient in beclin-1, a regulator of autophagy, are more susceptible to developing cancer and several human cancers have been associated with the loss of beclin-1 expression[459]. Speculatively, glycolipid loaded plasma cells in GD patients, due to the impaired function of autophagosomes, maybe less sensitive to chemotherapy.

GD osteoclast cultures generated more TRAP+ve osteoclasts at D21 than control osteoclast cultures and this was enhanced further by the co-culture (D14-21) of either NCI-H929 or U266 plasma cells. In this experimental system, the number of osteoclasts generated from controls remained low and did not increase post co-culture, suggesting that PBMCs cultured from GD patients are primed for osteoclastic differentiation. The co-culture of 5TGM1 or ARH-77 myeloma cells, which are reported to support osteoclastogenesis more vividly than the U266 cell line, may have led to enhanced osteoclast formation in control monolayers[434]. In the clinical setting, bony pathology, including lytic lesions, are often seen in patients with GD or myeloma[242;403]. Serum TRAP activity, a biomarker of osteoclast function, has been reported to be elevated in patients with GD[86]. Patients with GD or multiple myeloma have been shown to have raised serum levels of the osteoclast stimulating cytokines, MIP1α and MIP1β[79;247]. In particular, MIP1β has been associated with the severity of skeletal disease in GD[79]. Bone marrow cells, derived from myeloma patients, generated more osteoclasts in culture, compared to healthy controls, when supplemented with the conditioned growth medium of myeloma cell lines. In addition, the authors demonstrated a correlation between myeloma disease severity and the number of osteoclasts generated *in vitro*[305]. Cancer patients with bone involvement generate more osteoclasts in culture from PBMCs compared to healthy controls[290] . The number of osteoclasts generated *in vitro* has been shown to be predictive of skeletal metastasis in lung cancer patients[461]. Therefore, immature precursors from myeloma or cancer patients, like GD patients, are also primed for osteoclastic differentiation. In the data reported here, the addition of ERT prevented

osteoclastogenesis in GD contact co-cultures wells, with the number of osteoclasts similar to that generated in control co-cultures.

Unlike other investigators, these experiments were unable to demonstrate a contact-dependent increase in the number of osteoclasts generated from co-culture in non-GD patients[252;259;302;304]. This may be due to differences in methodology. In comparison, Yaccoby *et al*. (2004) used PBMCs mostly derived from myeloma patients and co-cultured them with primary plasma cells. Additionally, they used higher concentrations of RANK-L, supplemented their culture medium with dexamethasone during the first few days of culture and performed co-culture with a 20:1 plasma cell/osteoclast ratio[259]. Abe *et al*. (2004) used rabbit derived bone marrow stroma, higher concentrations of RANK-L, used a 1:2.5 plasma cell/osteoclast ratio and counted the number of resorption pits, in order to quantify osteoclastogenesis[252]. Hecht *et al*. 2008 used low concentrations of RANK-L (25ng/ml), co-cultured with a 1:4 to 1:1 plasma cell/osteoclast ratio and quantified osteoclastogenesis based on the detection of TRAP5b within the culture supernatant[302]. Unlike these investigators, the experiments reported in this chapter used RPMI 1640 rather than αMEM as the culture medium. Co-culture was initiated at a similar period to other investigators using PBMCs[259;302] but in comparison only $3x10^4$ NCI-H929 cells or $7.5x10^4$ U266 cells were added to D14 OC cultures (a total of $0.25x10^6$ plated on D0). Therefore, it may be that there were insufficient plasma cells in contact with the control adherent monolayer to enhance osteoclastogenesis during the initial and perhaps critical phases of co-culture. This assumption is supported by the following pieces of evidence:- (1) The supplementation of conditioned medium from myeloma cell lines has been demonstrated to prevent the apoptosis of rabbit osteoclasts in culture[298] (2) *In situ* hybridization and RT-PCR has demonstrated the expression of RANK-L, a potent stimulator of osteoclastogenesis, by both the NCI-H929 and U266 plasma cells lines[462], although this has been contested by other investigators[463] (3) Conditioned medium, NCI-H929 or U266 cell line derived, has been shown to enhance osteoclastogenesis in mouse derived bone marrow cell cultures[462].

The phagocytosis of plasma cells was noted in the majority of co-culture wells. GD monocytes cultured with heat denatured erythrocytes have been shown to

demonstrate tubular structures resembling glucosylceramide within the endo-lysosomal compartment, on electron microscopic examination[464]. Speculatively, the phagocytosis of plasma cells by GD monocytes could impair lysosomal function further and potentate their inherent ability to undergo osteoclastic differentiation. GD co-culture experiments demonstrated a similar increase in the number of D21 TRAP+ve osteoclasts, irrespective of contact, compared to GD non co-culture for either myeloma cell line, suggesting a soluble factor. This is in keeping with data cited above, where conditioned medium (CM) derived from NCI-H929 or U266 cells supported osteoclastogenesis[462]. Candidate cytokines supporting osteoclast generation in the culture supernatant include MIP-1α, MIP1β, IL-3, hepatocyte growth factor (HGF) and RANK-L[300]. Neutralizing antibodies to osteoclastogenic cytokines (IL-1α, IL-1β, IL-6, TNF-α, TNF-β and PTH-rp) failed to prevent bone lysis by the CM of 5TGM-1 myeloma cells in a foetal rat bone assay[434]. The inhibition of M1P-1α or RANK-L has led to decreased osteoclast production in a co-culture based model[259;305]. In contrast, to the results reported in this chapter, other investigators have reported a contact-dependent increase in osteoclastogenesis mediated by the VLA-4/VCAM-1 axis between plasma cells and the adherent monolayer[304;434]. Data suggest that MIP-1α and MIP1β, via an autocrine/paracrine mediated process, can facilitate the VLA-4 dependent adhesion of myeloma cells to VCAM-1 expressing stromal cells[304].

In conclusion osteoclastogenesis, based on the number of TRAP+ve cells, is enhanced in GD co-culture, irrespective of contact and is prevented by the addition of ERT. Further experiments are required, including immunofluorescence for the vitronectin receptor and functional assays of bone resorption, to confirm these initial observations. The various intracellular signalling pathways of osteoclastogenesis mediated by RANK-L and M-CSF binding should be explored to document more fully differences between GD patients and healthy controls.

Here, U266 cells were pre-treated with doxorubicin for 6 hours prior to plating alone or in contact co-culture (D18-21) for 3 days with GD or control-derived monolayers. Macrophage, stromal or OC monolayers have been suggested to protect primary plasma cells or myeloma cells lines from chemotherapy-induced death[245;252;303;446].

Compared to U266 culture alone, co-culture led to a lower number of recoverable plasma cells, although there were no difference between GD and control-derived monolayers at all doxorubicin concentrations. Giemsa staining of recovered plasma cells cultured alone revealed a large number of apoptotic bodies and a higher proportion of left shifted plasma cells with disrupted morphology compared to co-culture wells. In keeping with other investigators, the cultured monolayer was adherent to a more apoptotic population of non-harvestable plasma cells, compared to a relatively more healthy population in suspension[245]. The numerical deficit of harvested U266 cells from the co-culture experiments reported here was not explained by adherence alone and haemophagocytosis was demonstrated in the adherent monolayer. Zheng *et al*. (2009), the only published report on the co-culture of chemotherapy-treated plasma cells with macrophage monolayers, suggested protection against cell death in the absence of phagocytosis, based on the inhibition of endocytosis by cytochalasin B[245]. However, they did not conclusively demonstrate that cytochalasin B entirely inhibited phagocytic activity, as this analysis was only on the non-adherent fraction. In addition, it might be speculated that the morphological analysis of the adherent monolayer would have confirmed the engulfment of plasma cells. In the experiments reported in this chapter, despite a similar number of non-adherent annexinV/PI+ve cells for all doxorubicin treated wells (1-4μM), the percentage of TO-PRO+ve adherent cells increased dramatically with escalating drug concentration. This raises serious doubts about the conclusions of several investigators demonstrating a protective effect by osteoclasts, macrophages or bone marrow stroma based on the annexinV assay only[303;446].

GD monolayers, based on annexinV/PI data and 4 hour MTT dye reduction assay, led to a more apoptotic U266 plasma cell population compared to control co-culture (D18-21) in wells exposed to 4μM doxorubicin only, but not at lower concentrations. This suggests that GD osteoclast cultures do not provide an advantage in the rescue of doxorubicin treated U266 cells. This observation is only valid if GD adherent monolayers have equal phagocytic capacity and adhesive properties to apoptotic plasma cells at all tested concentrations of doxorubicin.

In conclusion, here, for the first time, several novel findings are reported of GD derived monolayers on plasma cell growth, survival and chemosensitivity. Co-

cultures derived from patients with GD primed plasma cells for survival against melphalan. Osteoclast cultures derived from GD patients did not convey a proliferative advantage over adherent monolayers generated from controls. GD macrophages were primed for osteoclastic differentiation and this was enhanced further by the co-culture of plasma cells. Further experiments are required with primary plasma cells or enzyme-deficient cell lines to replicate the *in vivo* microenvironment more accurately. More formal collaboration is required between individual treatment centres to collate patient data regarding the outcome of GD patients with plasma cell disorders. Currently it is unclear whether GD patients with myeloma have a worse prognosis or are more resistant to chemotherapy. Future work should build on the novel data presented above.

5 LYMPHOID ABNORMALITIES IN GAUCHER DISEASE

5.1 INTRODUCTION

Chapter 4 looked at whether the GD stromal environment promoted plasma cell growth, survival or harboured chemo-resistance. Locally, in the bone marrow, stromal cells and immune cells interact. GD is predominantly regarded as a macrophage disorder and the literature is relatively devoid of reports investigating the immunological microenvironment. There are only a handful of reports detailing lymphocyte abnormalities in GD[4;139;255;347;348;465]. Skewing of the immune profile in GD may facilitate plasma cell expansion, due to either impaired tumour surveillance or from the secretion of B-cell proliferating factors.

In this chapter it was hypothesised that patients with GD have abnormalities within their lymphocyte subsets, either numerical or functional, that impair tumour surveillance. Findings are compared to those described in non-GD patients with malignancies.

5.2 TUMOUR SURVEILLANCE

Tumour surveillance describes the hosts' ability to prevent, recognise and eradicate cancer cells. Impaired "tumour surveillance" may be as important mechanistically in the development of cancer as intrinsic changes within the cells themselves that confer malignant transformation. Observational data from patients with various cancers have demonstrated inferior outcomes or progressive disease in those with a low absolute lymphocyte counts[466;467], NK-cells[468], iNK-T cells[469] or elevated regulatory T-cells[470]. Additionally, an abnormal CD4/CD8 ratio has been shown to be a predictor of poor outcome in those with cancer[468]. Patients with human immunodeficiency virus (HIV) infection have an elevated risk of malignancy[471]. The stimulation of NK and iNK-T cells in mouse models of carcinogenesis has been shown to lead to tumour regression and prolonged survival[472]. Furthermore patients with common variable immunodeficiency, a condition characterised by both T- and B-cell defects, have an increased risk of

developing gastric and haematological cancers[473]. Dhodapkar *et al*. (2003) assessed the ability of plasma cell antigen presentation by dendritic cells (DCs) to stimulate autologous T-cells derived from patients with MGUS and myeloma. They showed that patients with myeloma had impaired effector T-cell responses, based on ELISPOT IFN-γ production, when compared to patients with MGUS[474]. These studies demonstrate the importance of lymphocytic cells in the immunological control of cancer.

Both the innate and adaptive immune system partakes in the immunological attack of cancer cells. Tumour-derived antigens are presented by cell surface MHC class I molecules to the T-cell receptor of cytotoxic T-cells (CTLs). Recognition of non-self leads to the lysis of cancer cells, secondary to the release of perforin, FasL, IFN-γ, granzymes and granulysin[336].

Stimulated iNK-T cells can kill cancer cells via the activation of CTLs and NK- cells[338]. NK-cell activity is modulated by inhibitory and activating cell surface receptors[475]. NK-cells can induce tumour cell death directly by the exocytosis of cytotoxic granules, the engagement of tumour cell death receptors (Fas/TRAIL) and indirectly by maturing dendritic cells, all of which are crucial in facilitating a potent CTL response[337]. Plasma cells are an important component of the adaptive immune system and generate a diverse array of antibodies recognising non-self. The production of tumour-specific immunoglobulins leads to cancer cell death secondary to direct opsonisation, complement-dependent cytotoxicity or antibody-dependent cytotoxicity, via the activation of NK-cells or macrophages[336]. Macrophage effector function has been suggested to be compromised in patients with GD, with defects in free radical production, phagocytosis and the killing of staphylococcus aureus being reported[476]. Lytic granules, including perforin, are stored within lysosomal-like compartments and their release maybe impaired in GD[477].

5.2.1 NK-CELLS

NK-cells play a major role in innate immunity and kill tumour cells via the release of cytotoxic granules or from the engagement of trans-membrane death receptors, including

Fas and TRAIL. NK-cells are typically CD3-ve, CD56+ve and CD16+ve and constitute approximately 15% of peripheral blood lymphocytes[475]. Two distinct populations can be identified based on their intensity of CD56 expression. The CD56 dim population predominates in peripheral blood (~90%) and confers high cytotoxicity. In contrast, the CD56 bright population is less common, secretes high quantities of cytokine following activation but confers little direct cytotoxicity[337]. NK cells, like T-cells, have an array of inhibitory and activating receptors including killer immunoglobulin-like receptors (KIRs), lectin receptors, leukocyte immunoglobulin-like receptors, lectin receptors, Fc receptors and MHC class I molecules[475]. Tumour cells can evade elimination by CTLs by the down-regulation of MHC class I molecules. However the loss of MHC class I expression in cancer cells induces killing by NK-cells due to loss of inhibitory receptor signalling[337;475]. Low NK-cell activity has been correlated with an elevated risk of cancer development and the subsequent progression to metastatic disease[342;343].

5.2.2 INVARIANT NK-T CELLS

Although iNK-T cells constitute 0.01-1% of all peripheral blood T-cells[478], they play a key role in innate and adaptive immunity. Animal models have demonstrated their immunological importance in regulating cancer surveillance, allergic reactions, inflammatory conditions, auto-immune disease, microbial defence and organ rejection[338]. In peripheral blood, their numbers have been found to be depleted in untreated patients with either melanoma or prostate cancer[479;480]. Similarly to conventional T-cells, they have a T-cell receptor but in contrast, they uniformly express the same alpha chain (Vα24Jα18 humans; Vα14Jα18 mice)[481] with preferentially pairing to Vβ11. Antigen presenting cells, including macrophages and DCs, present processed glycolipid on their surface by CD1d tetramers. iNK-T cells are activated by the engagement of their T-cell receptor by CD1d presented antigens[332]. CD1d molecules are related to MHC class I molecules and are complexed with β2 microglobulin. Elevated surface CD1d expression has been demonstrated on the monocytes of patients with GD[4].

Glycolipids have important T-cell immunomodulatory effects[21]. The administration of beta-glycolipids (β-glucosylceramide being an example) has been shown to beneficially effect the T-cell profile in various animal models of disease including hepatitis[482], colitis[483], hepatocellular carcinoma[21] and acute/chronic GvHD[484]. Alpha glycolipids, such as alpha-galactosylceramide, also alter T-cell subsets, ameliorate disease and alter cytokine profiles[485]. Zigmond *et al.* (2007) administered glucosylceramide to mouse models of hepatocellular carcinoma (HCC) and autoimmune colitis. Increased IFN-γ production was associated with tumour regression in the HCC mouse model whereas depressed IFN-γ levels were documented in mice with auto-immune colitis[483]. Therefore, iNK-T cells have remarkable plasticity, expressing either a Th1 (IFN-γ) or Th2 (IL-4) cytokine profile depending on the net contribution of individual stimuli. Although glucosylceramide, a naturally occurring sphingolipid, has been shown to have an anti-inflammatory and anti-tumour effect in mouse models, it is currently unknown whether β-glycolipids are effectively loaded onto CD1d molecules or out-compete more potent immuno-modulatory ligands in GD.

5.2.3 HYPOTHESIS

The following hypotheses are explored in this chapter:-

Hypothesis 1:- GD lymphocytes have low glucocerebrosidase activity, accumulate substrate and have abnormalities within their lymphoid repertoire that contribute to a microenvironment favourable to carcinogenesis.
Rationale:- Non-GD patients with abnormalities in their lymphocyte count or subsets, including NK-cells, have an increased incidence of malignancy.
Methods:- Local patient data, immunophenotyping and electron microscopy.

Hypothesis 2:- Patients with GD, due to disturbances in sphingolipid metabolism and glycolipid presentation, have abnormalities in iNK-T cell expansion. This contributes to a microenvironment conducive to malignancy.
Rationale:- Numerical low numbers of invariant NK-T cells are seen in individuals with malignancy.

Methods:- Sphingolipid stimulation assays, cell culture.

Hypothesis 3:- Patients with GD, due to impaired NK-function, have impaired tumour surveillance.

Rationale:- Decreased peripheral NK-cell lytic activity is associated with impaired tumour surveillance in non-GD individuals.

Methods:- NK killing assays.

Hypothesis 4:- Patients with GD have numerical and function abnormalities within their peripheral blood cells that lead to impaired tumour surveillance.

Rationale:- Non-GD patients with impaired peripheral blood killing assays have an elevated risk of malignancy.

Methods:- Peripheral blood killing assays.

5.3 METHODS

The methods described here are supplementary to those described in chapter 2.

5.3.1 FLOW CYTOMETRY – LYMPHOCYTE SUBSETS

Flow cytometry was performed, as described in section 2.5, in order to establish the percentage of CD3+ve, CD3-ve/CD56+ve, CD3+ve/CD56+ve, CD3+ve/6B11+ve, CD3+ve/CD4+ve and CD3+ve/CD8+ve lymphocytes in healthy controls and patients with GD. Lymphocytes were identified based on their forward and side-scatter characteristics with a minimum of 10,000 gated events acquired.

5.3.2 ANALYSIS OF B-CELL SUBSETS

Peripheral blood B-cell subsets were analysed by staff in the Department of Immunology at the Royal Free Hospital (London, UK) according to a standard operating procedure. Briefly, 1ml of sample blood (EDTA tubes) was added to 14mls PBSA (5l PBS, 10g azide, 10g bovine serum albumin). Cells were centrifuged at 1500rpm for 15 minutes and re-suspended in a 1:10 dilution of Hoffmans buffer (8.3g NH_4Cl, 1g $HC0_3$, 37mg disodium EDTA, pH7.4). Following incubation at room temperature for 10 minutes, cells were centrifuged for 5 minutes at 1500rpm. Cells were washed and re-suspended in 1ml PBSA. 100µl of cell suspension was added to two tubes containing the following antibody cocktails:-

Tube A:- CD27-FITC (Beckham Coulter) 1/5, CD19-PC7 (BD Biosciences) 1/4, IgD-PE (Oxford Biotechnology) 1/200 and IgM-Cy5 (Jackson Immuno Research) 1/400.
Tube B:- CD38-FITC (BD Biosciences) 1/4, CD21-PE (BD Biosciences) 1/4, CD19-PC7 1/4 and IgM-Cy5 1/100.

Each tube was made up to a total volume of 20µl with PBSA. Samples tubes were mixed thoroughly and incubated in the dark for 15 minutes. 20µl of 1% paraformaldehyde was added to each tube prior to acquisition on a FACSCalibur (Becton Dickinson). Individual B-cells subsets were quantified as detailed in Figure 5-1.

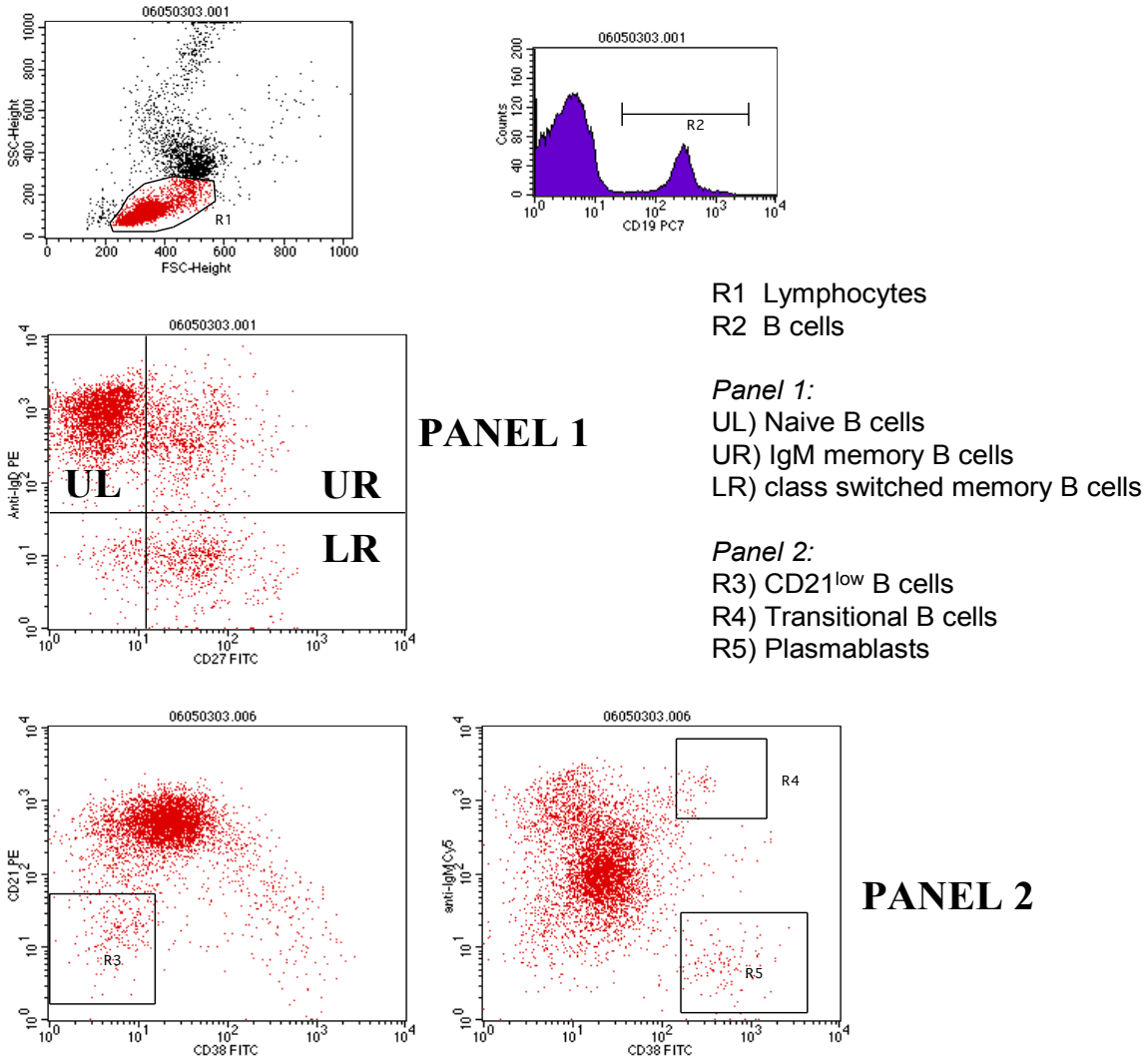


Figure 5-1. B-cell subsets determined using two panels of antibodies.

5.3.3 CONDURITOL-B EPOXIDE ASSAYS

PBMCs were separated from peripheral blood as described in section 2.4.1. Ce1ls ($1x10^6$/ml) were plated, in duplicate. Growth medium medium was supplemented with IL-2 (200iu/ml) and several different concentrations of CBE (0-500μM). A half change in growth medium was performed on D4. PBMCs were recovered on D8 and glucocerebrosidase activity was determined using a fluorometric-based assay (chapter 2.8.5). CBE was stored in concentrated aliquots (100μL; 50mM; -20°C) until required.

IL-2 was reconstituted in 50mM acetic acid (filtered) and divided into aliquots of 10,000U (stored at -20°C until use).

The cytotoxicity of CBE was determined by plating $2x10^5$ PBMCs, in triplicate, in 96-well plates. Growth medium was supplemented with IL-2 (200iu/ml) and several different concentrations of CBE (0-500μM). Distilled water (300μL) was placed into empty wells to prevent sample evaporation. Culture wells underwent a half change in growth medium on D4 (100μL) and an MTS dye reduction assay was performed on D7 as described in section 2.8.3.

5.3.3.1 PBMC LIPID STIMULATION CULTURES

PBMCs from healthy controls and patients with GD were separated from peripheral blood using 3% dextran and Ficoll-Paque™ plus, as described in section 2.4.1. The percentage of 6B11+ve lymphocytes in a CD3 gate was quantified to establish the percentage of iNK-T cells. The 6B11 monoclonal antibody identifies the CDR3 epitope on the TCR α-chain formed by recombination of Vα24 and Jα18. As described in chapter 2.5, cultured cells were incubated for 30 minutes on ice with antibodies against 6B11 (FITC) and CD3 (APC).

2mls of PBMCs were plated ($1x10^6$/ml) in 24-well plates and cultured in R10 with 200iu/ml IL-2 alone or supplemented, in addition, with either 100ng/ml of alpha-galactosylceramide (αGalCer; Enzo Life Sciences; distributed Axxora, Nottingham, UK) or 100ng/ml of isoglobotrihexosylceramide (iGB3; Enzo Life Sciences). Both glycolipids were soluble in pyridine and stored concentrated at 5μg/ml at -20°C until use. PBMCs were cultured for 14 days with twice weekly half changes in growth medium (1ml). Glycolipid was added to wells at 100ng/ml for the first two medium changes only. The percentage of iNK-T cells (CD3+ve/6B11+ve) was quantified on D14. Recovered cells were identified based on the expression of the pan T-cell marker, CD3, within a live gate. The majority of cultured cells were T-cells and many IL-2 stimulated cells were apoptotic as indicated by the dense population of cells with low forward scatter. A minimum of 50,000 CD3+ve events were enumerated with the

additional criteria of detecting at least 100 cells with CD3/6B11 positivity. 6B11+ve iNK-T cells co-expressed CD3 as demonstrated in Figure 5-2.

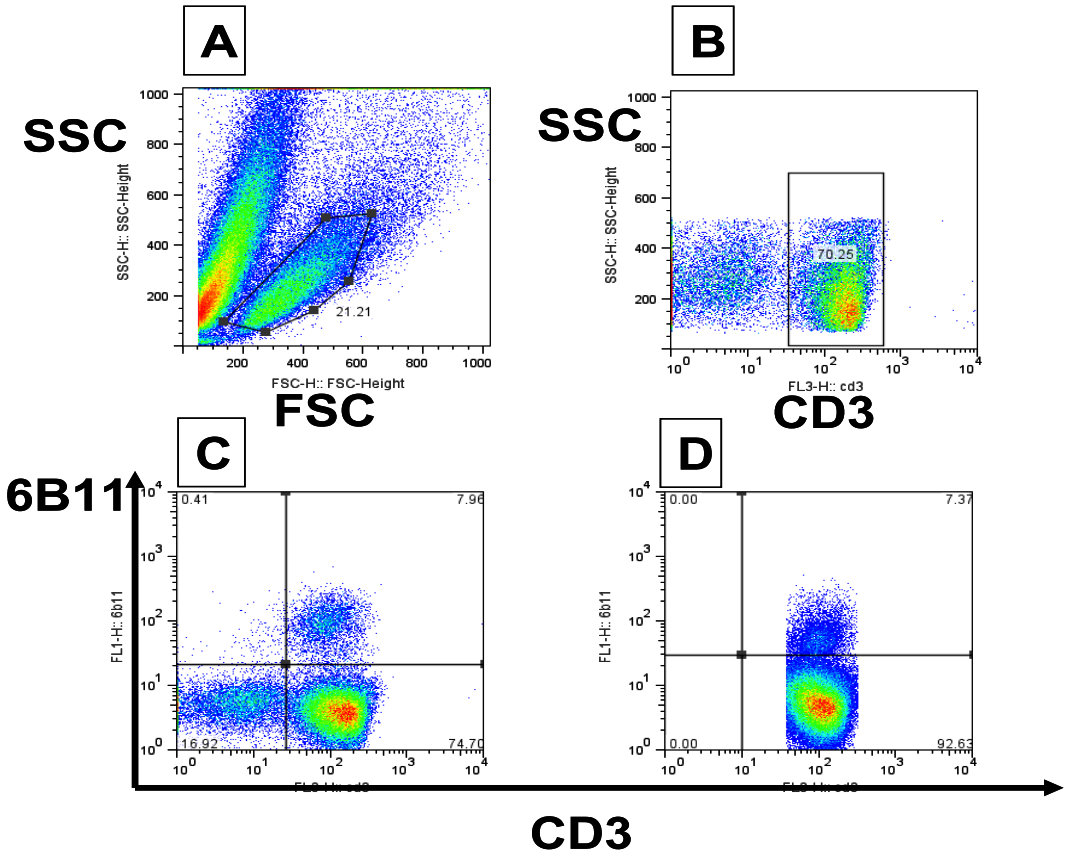


Figure 5-2. Flow cytometry for invariant NK-T cells.
6B11+ve invariant NK-T cells were identified post 14 day culture (αGalCer supplemented) within a live gate and reported as a % of the total number of CD3+ve cells (A, B, D). (C) Type I NK cells co-expressed CD3.

5.3.3.2 PBMC αGALCER - CBE CULTURES

PBMCs from healthy controls were cultured as described above (see section 5.4.3.1), but in contrast cultures were terminated on D10. Experiments were performed in duplicate and cells were grown in IL-2 alone (200iu/ml), IL-2 plus αGalCer (100ng/ml) or with IL2, αGalCer and CBE (50µM or 100µM). The percentage of iNK-T cells was determined on D10 as described in the section above.

5.3.4 LYMPHOCYTE GLUCOCEREBROSIDASE ACTIVITY

Flow cytometry was used to determine enzymatic activity in control and GD- derived lymphocytes as described in chapter 2.5.4 (FDGlu assay). Within the lymphocyte gate, a minimum of 10,000 events were analysed with at least 2,500 events in lymphoid sub-populations. The assay was made specific for lysosomal glucocerebrosidase by dividing the MFI of the sample, reflecting liberated fluorescein from the carrier substrate di-ß-D-glucopyranoside, by that obtained from a paired sample pre-treated with the inhibitor CBE.

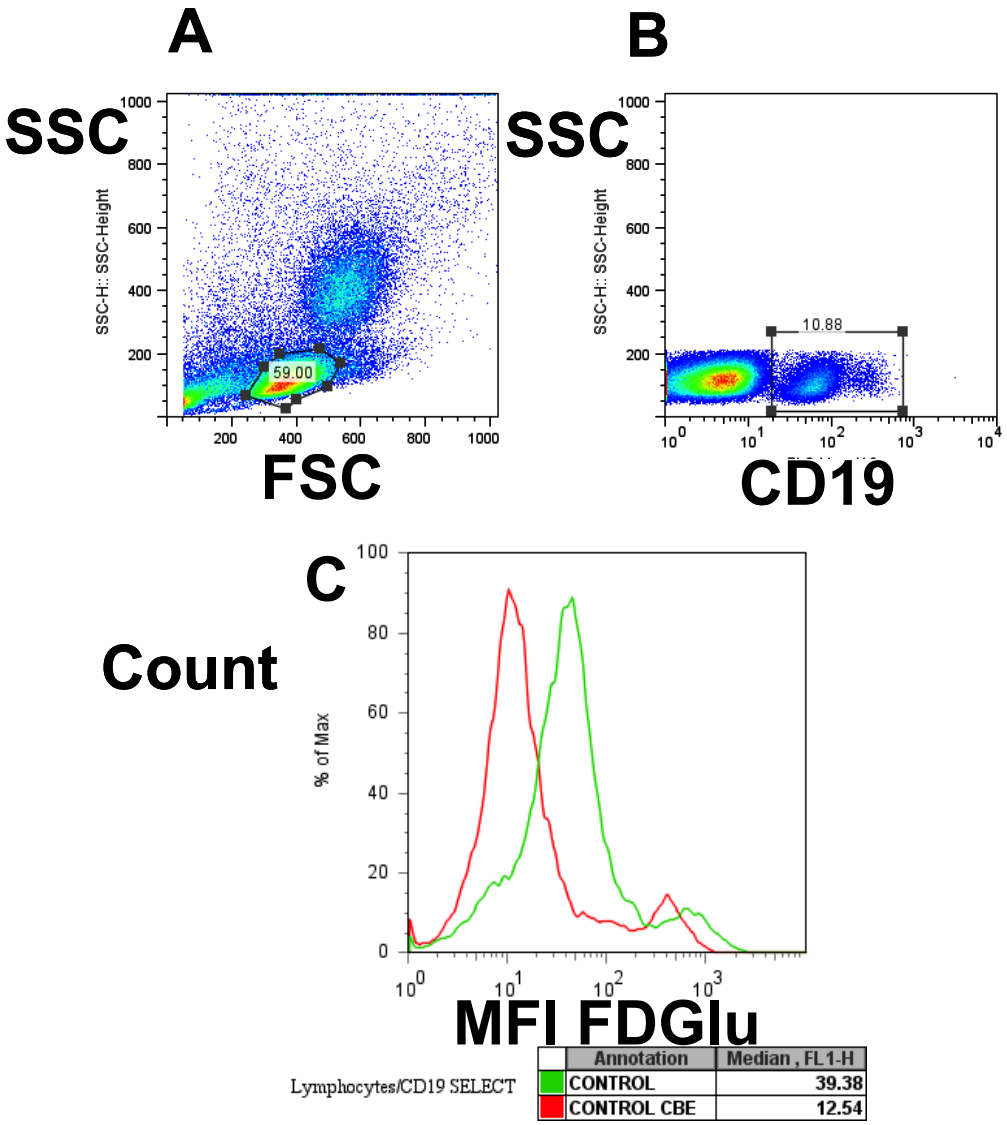


Figure 5-3. (A, B) Flow cytometry plots demonstrating the gating of CD19+ve lymphocytes. (C) Representative histograms demonstrating liberated fluorescein from control CD19+ve lymphocytes (green line) compared to a paired sample pre-treated with CBE (red line).

5.3.5 NK-CELL AND PBMC KILLING ASSAYS

NK-cell killing assays were performed as described in detail in section 2.11. Briefly, NK cells were separated from the peripheral blood of healthy controls and patients with GD using MACs technology, as described in section 2.11.1. Isolated CD56+ve NK cells were cultured alone or stimulated with IL-2 (200 iu/ml) overnight. Killing assays were performed using a 5:1 effector:target ratio. Target cells (NCI-H929, JJN3 and K562 cell lines) were pre-labelled with the fluorescent dye PKH26. K562 is a monocytic leukaemia-derived cell line and is particularly sensitive to effector cell killing[342;343;486]. The percentage of apoptotic PKH26 target cells was determined by uptake of the nucleic acid dye TO-PRO®-3-iodide (see chapter 2.5.5) on completion of the 4 hour killing assay.

All PBMC killing assays were stimulated overnight with IL-2 (200iu/ml) and performed at effector:target ratios of 40:1, 20:1 and 10:1. The percentage of TO-PRO+ve target cells (NCI-H929, JJN3 or K562) was determined 4 hours post co-culture.

Paired healthy controls were within 10 years of age to those with GD and performed in parallel at the same time.

5.3.6 ELECTRON MICROSCOPY

PBMCs were isolated from peripheral blood and prepared for electron microscopy as described in chapter 2.4.2. Prepared samples were viewed using a Phillips CM120 transmission electron microscope.

5.3.7 IMMUNOFLUORESCENT STAINING

Cytospins were prepared using PBMCs isolated from healthy controls and patients with GD ($0.22x10^6$/ml; 400rpm; 5 minutes). Cytospins were dried in air for 2 hours and slides were stained with lysotracker and BODIPY-tagged lactosylceramide, as described in chapter 2.12. Slides were analysed using a Zeiss LSM 510 Meta confocal microscopy.

5.4 RESULTS

5.4.1 LYMPHOCYTE COUNT

It has been suggested that patients with GD may develop gammopathy on a background of chronic antigenic stimulation. This may be reflected by an increased peripheral lymphocyte count, perhaps representing polyclonal stimulation. The mean ALC was similar between demographically matched healthy controls ($1.6\pm0.4x10^9$/l; mean±SD; n=22), untreated patients with GD (n=45; $1.7\pm1.2x10^9$/l) and from the same patients (n=45) after receiving a minimum of 12 months ERT ($1.9\pm0.9x10^9$/l). Within the ERT cohort, 6/8 patients with the highest lymphocyte count had undergone splenectomy (Boxed group; Figure 5.4) and this difference was statistically significant when compared to the number of asplenic individuals outside of the boxed group (Fisher's exact test, $p<0.01$). The development of polyclonal lymphocytosis is not uncommon in patients without GD following splenectomy and therefore this is an important confounding factor.

The mean lymphocyte/WCC ratio was identical between individual groups, although, in the ERT cohort the spread of results was clearly greater. Paired analysis failed to demonstrated a reduction in the lymphocyte count following a minimum of 12 months ERT (Pre ERT, $1.8\pm1.3x10^9$/l; ERT $1.9\pm0.9x10^9$/l; n=31; mean±SD). This is in contrast to absolute immunoglobulin levels, which decreased with therapy (see Chapter 3). In addition, the mean ALC was similar in untreated individuals with gammopathy ($1.7\pm1.0x10^9$/l; n=25), polyclonal or monoclonal, and those with normal immunoglobulin analysis ($1.7\pm1.5x10^9$/l; n=19).

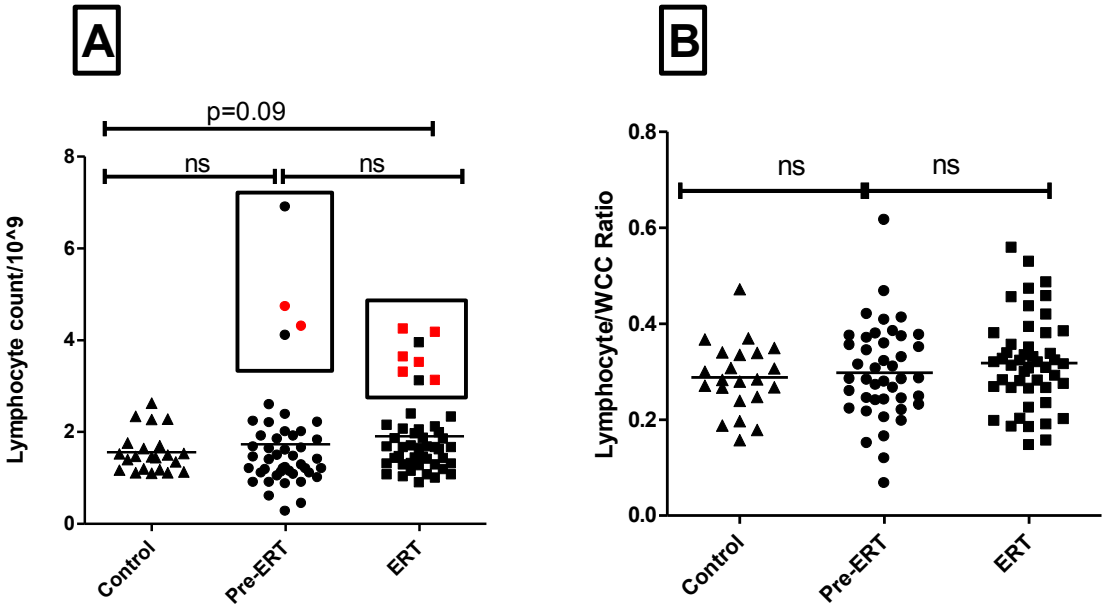


Figure 5-4. (A) Absolute lymphocyte count and (B) lymphocyte/white cell count ratio in healthy controls, untreated GD patients and those on ERT.
Mean indicated by horizontal line. Boxed populations in (A) represent outliers and individuals who have had a splenectomy are represented in red.

5.4.2 FUNCTIONAL AND STRUCTIONAL ABNORMALITIES IN GD LYMPHOCYTES

Peripheral lymphocytes are young haematopoietic cells with a short half-life in blood. Therefore, they may not develop the same functional abnormalities or ultra-structural characteristics as lipid-laden macrophages. Experiments in this section investigate glucocerebrosidase activity, glucosylceramide deposition and lipid trafficking within individual lymphocyte subsets.

5.4.2.1 LYMPHOCYTE GLUCOCEREBROSIDASE ACTIVITY

Peripheral blood monocytes and lymphocytes were gated on their forward and side scatter characteristics. Glucocerebrosidase activity was determined by the MFI of liberated fluorochrome from the carrier substrate, FDGlu, and was expressed as a ratio of the sample MFI to that obtained from a paired sample from the same individual treated with the lysosomal glucocerebrosidase inhibitor CBE. Lymphocytes, compared to monocytes, have relatively low glucocerebrosidase activity (Figure 5-5). However

lymphocytes from patients with GD demonstrated a significantly lower enzyme activity compared to controls ($p<0.01$).

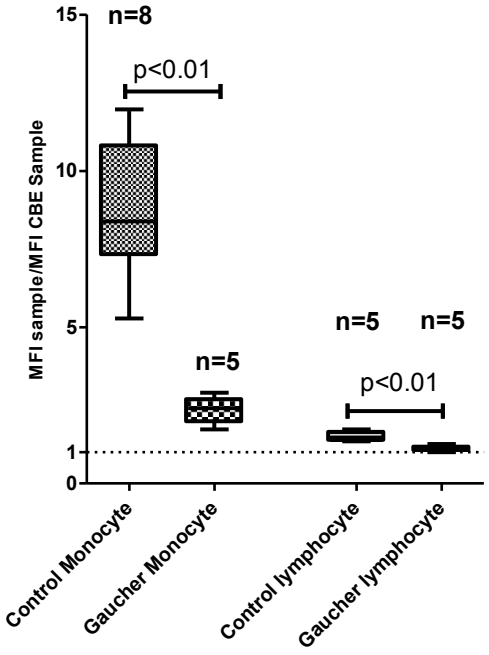


Figure 5-5. Glucocerebrosidase activity as determined by flow cytometry of monocytes and lymphocytes isolated from patients with GD or healthy controls.

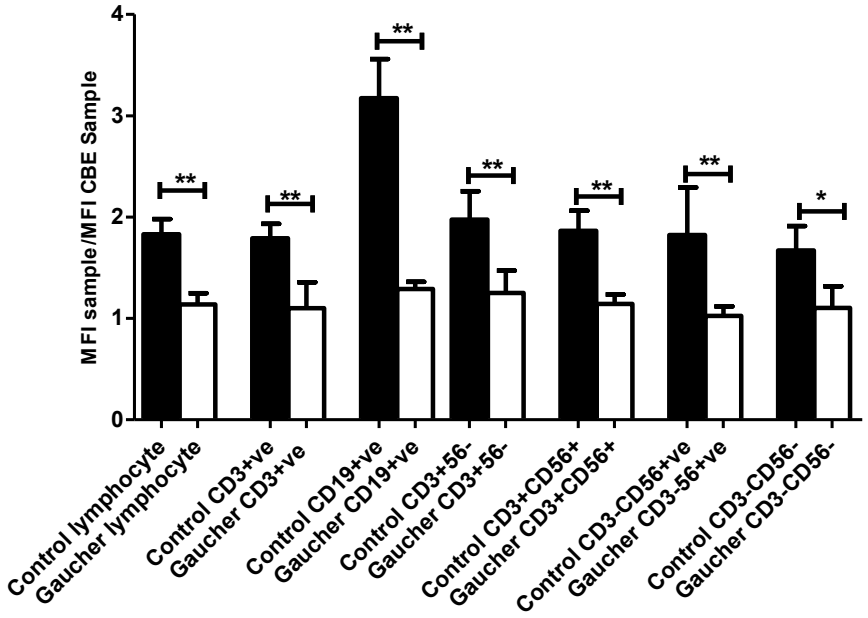


Figure 5-6. Glucocerebrosidase activity as determined by flow cytometry of lymphocyte subsets from healthy controls or patients with GD (Control n=5, GD n=5).
***p<0.05 **p<0.01**

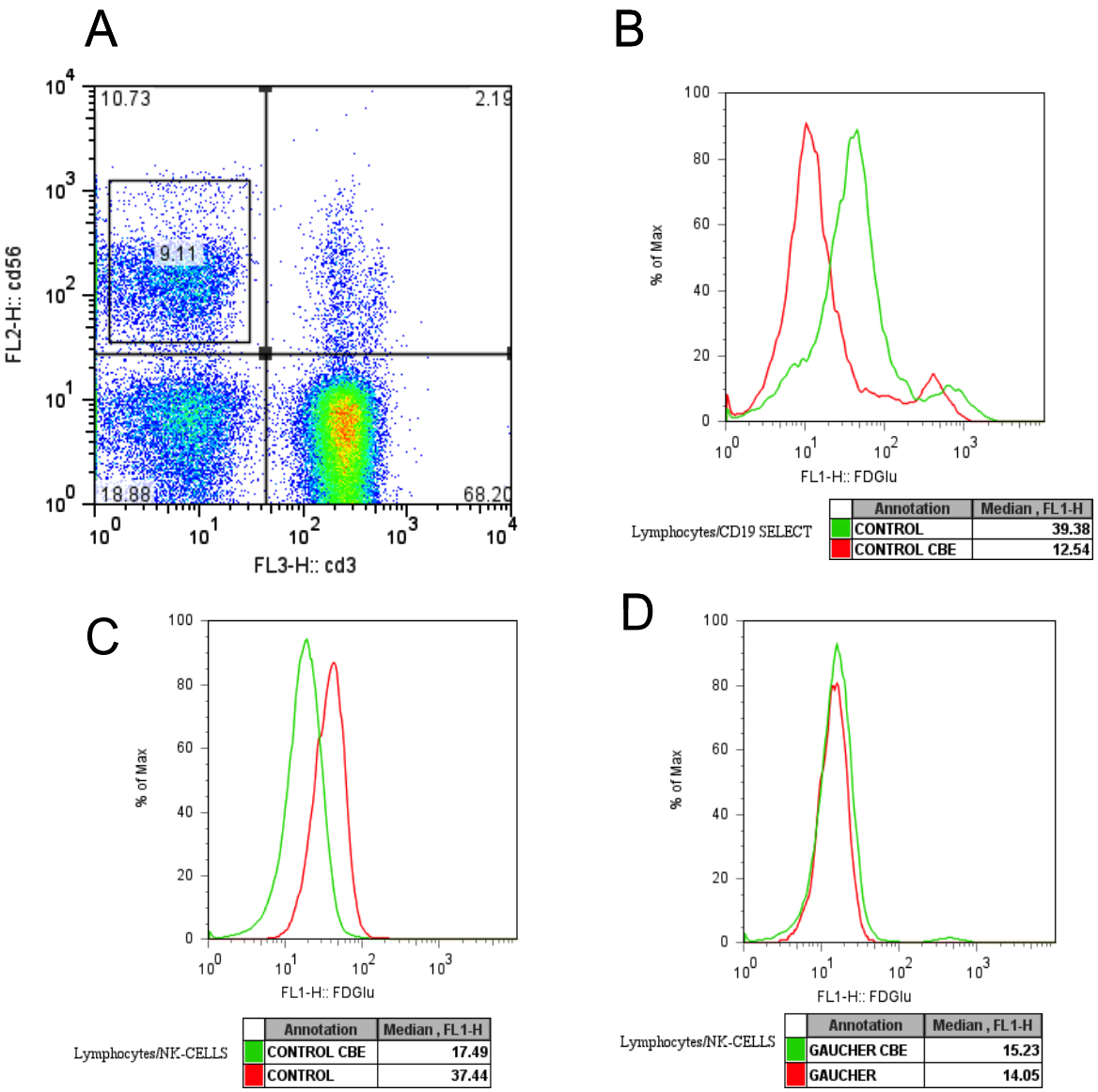


Figure 5-7. Lymphocyte glucocerebrosidase activity (Control versus Gaucher).
(a) Glucocerebrosidase activity in lymphocyte subsets was determined by gating on individual populations using fluorochromes detectable in FL2/FL3. (B, C) Histogram plot of FDGlu activity in FL1 of B and NK cells from healthy controls. (D) Histogram demonstrating absent activity in NK cells derived from Gaucher patients.

Individual lymphocyte subsets were identified by immunostaining and the enzyme activity within each subset was quantified. CD19+ve B-cells, compared to CD3+ve T-cells, had significantly higher glucocerebrosidase activity in healthy controls ($p<0.01$; MW test). Enzyme activity was significantly lower in all lymphoid subsets derived from patients with GD. (Figure 5-6; Figure 5-7).

5.4.2.2 ABNORMAL MEMBRANE TRAFFICKING IN GD LYMPHOCYTES

It has previously been shown that glucocerebrosidase-deficient cells have impaired re-cycling of membrane lactosylceramide within the endo-lysosomal compartment [487]. Lactosylceramide is normally concentrated in membrane structures with a small amount being recycled in the endocytic pathway. Previously, our laboratory has demonstrated the abnormal accumulation of lactosylceramide in lysosomes of cultured macrophages from patients with GD (Personnel communication; Dr Derralynn Hughes and Matthew Reed).

Immunofluorescent staining was used to assess the distribution of BODIPY lactosylceramide (visualised green) within the cell, including the endo-lysosomal compartment (Lysotracker; visualised red). In both monocytes and lymphocytes derived from healthy controls, lactosylceramide was concentrated in the Golgi and endosomal membranes (Figure 5-8). Compared to control lymphocytes, control monocytes had significantly more lysosomes, as demonstrated by the number of lysotracker stained organelles. Both GD monocytes and lymphocytes accumulated lactosylceramide in the lysosomal compartment, as demonstrated by the co-localisation of BODIPY (visualised yellow). Thus, it can be concluded that lymphocytes from patients have a pattern of lipid trafficking consistent with lysosomal dysfunction.

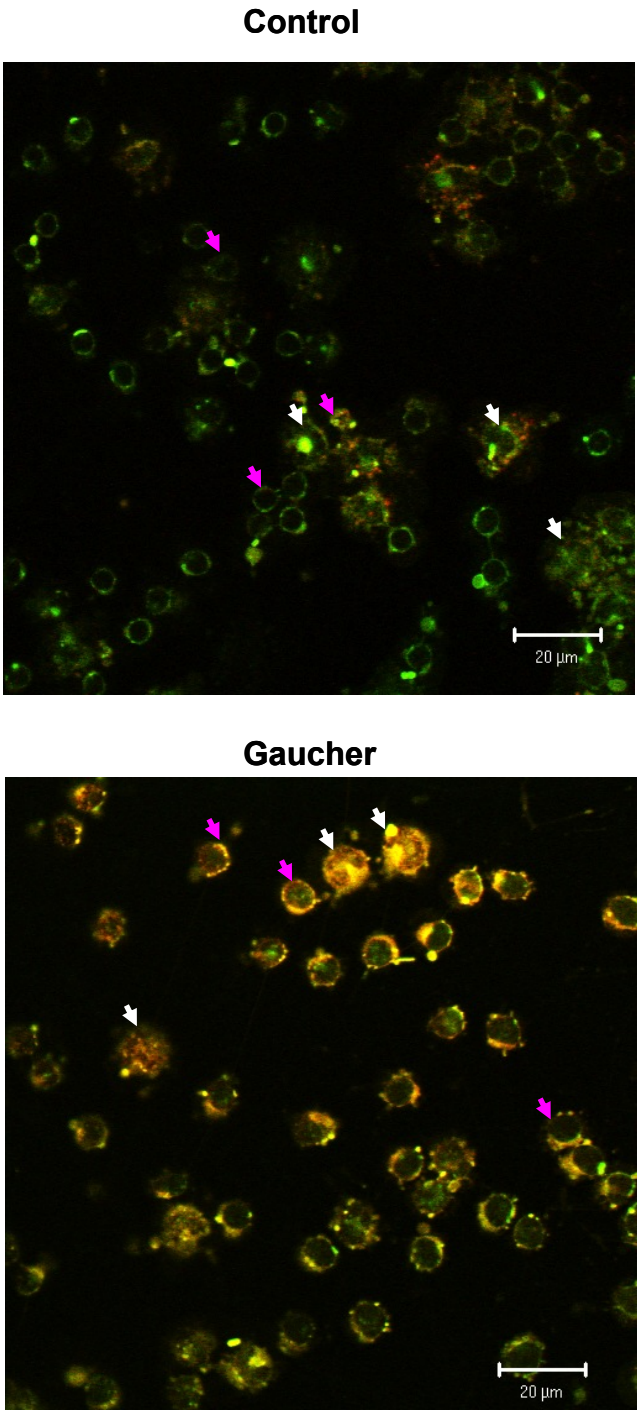


Figure 5-8. Representative immunofluorescent images of PBMCs from patients with GD and healthy controls.
Patients with GD concentrated BODIPY-lactosylceramide (green) to the lysosomal compartment (red) as visualised yellow (combined signal). In contrast, cells from healthy controls rarely co-localised signals. Purple arrows indicate lymphocytes, white arrows represent monocytes.

5.4.2.3 ELECTRON MICROSCOPY OF LYMPHOCYTES

It has been demonstrated above that lymphocytes from patients with GD have low glucocerebrosidase activity and dysfunctional lysosomes. Using transmission electron microscopy (TEM), the lymphocytes of 3 patients with GD, including one untreated, were assessed for evidence of glucosylceramide accumulation. Accumulated glucosylceramide adopts a tubular structure when deposited in the lysosomes of enzyme-deficient macrophages[264].

Lymphocytes were identified on TEM based on size, shape and a small rim of peri-nuclear cytoplasm. In contrast, monocytes were larger, had a lower nuclear:cytoplasmic ratio, contained more organelles and had an indented nucleus. Several mitochondria were seen in the cytoplasm of lymphocytes, but the presence of other identifiable organelles was rare. There was no difference in the morphology of lymphocytes derived from healthy controls or patients with GD. Distended structures, consistent with engorged lysosomes, were not detected in lymphocytes from any of the three GD patients, despite scanning many slices. In addition, accumulation of tubular structures within the organelles of GD monocytes was not demonstrated (data not shown). The ultra-structural appearances of monocytes derived from GD patients were identical to those seen in healthy controls.

5.4.3 B-CELL SUBSETS

Building upon the theory of chronic antigenic stimulation, it was hypothesised that patients with GD, due to their elevated incidence of gammopathy, may have a relatively higher percentage of differentiated B-cells in peripheral blood compared to healthy controls. The control population was a historical cohort consisting of 25 healthy volunteers. The cohort of GD patients (n=10) described below were of mixed clinical severity, with 8/10 patients receiving ERT.

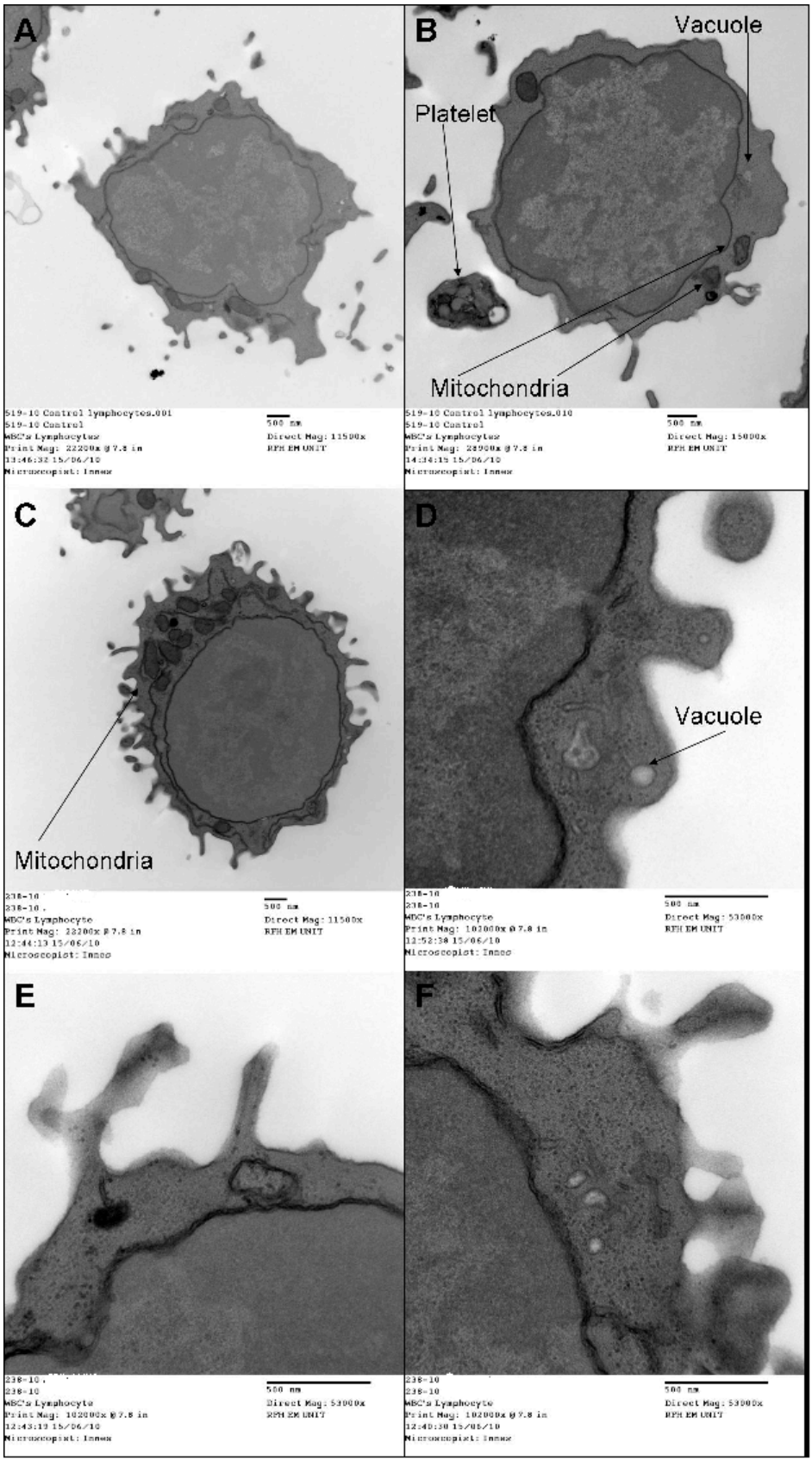


Figure 5-9. Transmission electron microscopy of lymphocytes derived from controls (A, B) and an untreated patient with GD (C-F).

	Immunophenotype	Control % Mean±SD	Gaucher % Mean±SD	p-value
Number	-	25	10	-
CD19	CD19+ve	12.0±4.9	9.3±3.2	0.13
Naïve B-cells	CD19+ve IgD+ve CD27-ve	64.9±12.8	63.9±14.4	0.84
IgM Memory	CD19+ve IgD+ve CD27+ve	12.0±6.9	12.7±5.6	0.65
Class Switched	CD19+ve IgD-ve CD27+ve	13.2±5.0	11.7±8.0	0.48
CD21 low B-cells	CD19+ve CD21-ve CD38-ve	7.4±2.5	8.7±3.5	0.52
Transitional B-cells	CD19+ve $CD21^{int}$ CD38+ve IgM+ve	5.3±2.4	7.0±3.5	0.25
Plasmablasts	CD19+ve CD21 –ve CD38+ve IgM-ve	2.2±2.1	0.8±0.7	**<0.05**

Table 5-1. Percentage of B-cell subsets in the peripheral blood of controls and Gaucher patients. Int = intermediate.

Patients with GD had a normal percentage of CD19+ve B-cells in the peripheral blood. Surprisingly, the number of plasmablasts was lower in patients with GD ($p<0.05$), including two individuals with a long-standing paraprotein. These results demonstrate that patients with GD do not have skewing of the B-cell population towards a more mature phenotype, although the majority were receiving ERT.

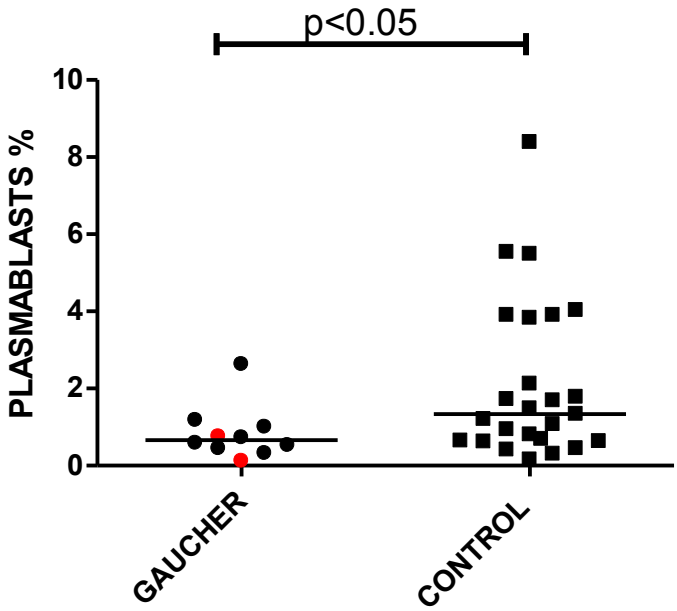


Figure 5-10. Percentage of peripheral plasmablasts in the blood of healthy controls and GD patients. Median indicated by horizontal line. GD patients with a paraprotein are highlighted in red.

5.4.4 T-CELL SUBSETS

GD has been linked to a higher incidence of several malignancies and abnormalities within T-cell subsets (as discussed in section 5.2), including the depletion of NK-cell numbers, may contribute to impaired tumour surveillance. Healthy control populations were age matched (described in Table 5.2) in preference to gender and race. There was a maximum of 3 patients who were not receiving ERT within any of the analysed groups. Patients with GD were mainly composed of middle-aged men who represent the available patient population undergoing regular review or infusional treatment in our centre.

Comparison	Control Age	Gaucher Age	Control Number	Gaucher Number	Control M:F Ratio	Gaucher M:F Ratio
CD3/CD56	41.7	46.6	31	32	1.2	1.9
CD3	42.3	46.9	24	21	1.7	3.3
CD3/CD4	41.7	46.7	37	31	1.5	1.8
CD3/CD8	38.8	45.0	32	28	1.9	2.1
CD1d Monocytes	42.4	44.6	17	19	1.1	2.1
6B11/iNKT	41.4	46.7	26	26	1.4	2.7

Table 5-2. Demographic data on GD patients and healthy controls for each comparison analysed below.

5.4.4.1 CD3 POPULATION

There were identical numbers of CD3+ve T-cells in healthy controls and GD patients.

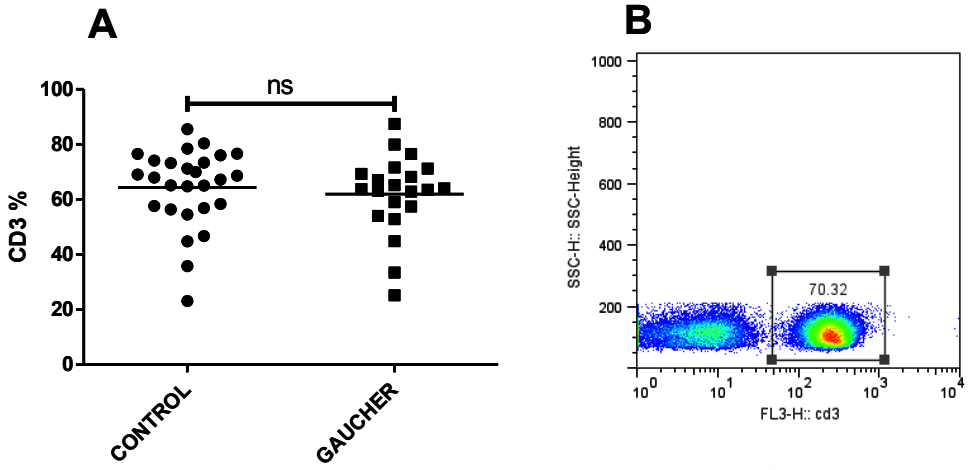


Figure 5-11. (A) Percentage of CD3+ve lymphocytes in controls and GD patients. Mean indicated by plotted line (B) Flow cytometric quantification of CD3+ve cells within a lymphocyte gate.

5.4.4.2 CD3/4 AND CD3/8 POSITIVE POPULATIONS

The percentage of CD3+ve/CD4+ve and CD3+ve/CD8+ve cells was determined within the lymphoid population (Figure 5-12).

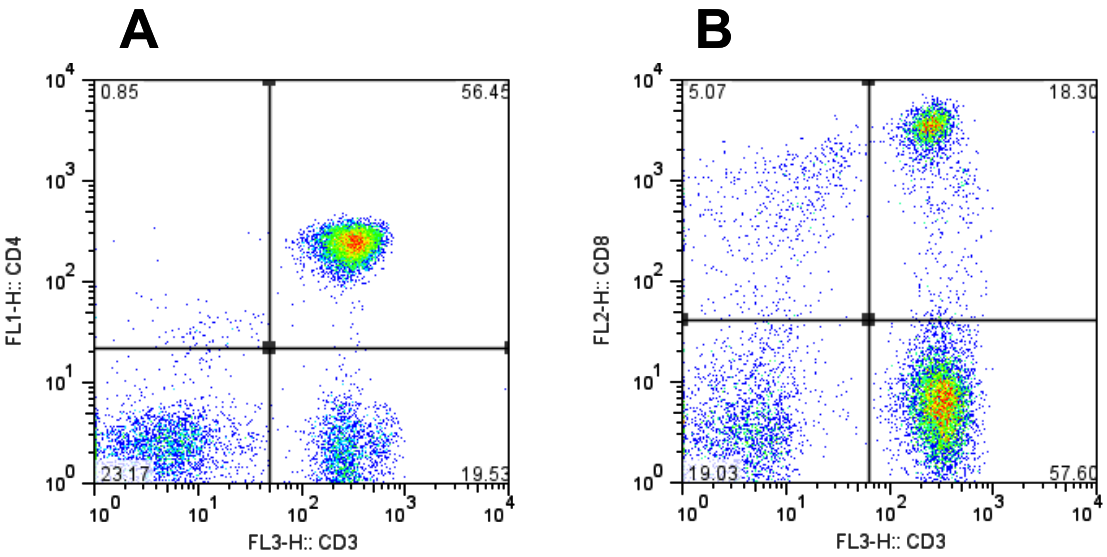


Figure 5-12. Representative flow cytometry plots of (A) CD3/4 and (B) CD3/8 lymphocyte populations.

Compared to the control group, GD patients had a significantly lower percentage of CD3+ve/CD4+ve lymphocytes ($p<0.05$). There were a decreased percentage of CD3+ve/CD4+ve cells ($p=0.01$) in splenectomised GD patients compared to the "unselect" GD population (Figure 5-13). Controls had a lower percentage of CD3+ve/CD8+ve cells ($p=0.05$) than GD patients with gammopathy. Compared to non-GD patients, the mean percentage of CD8+ve T-cells in the "unselect" GD population was not elevated. Patients with gammopathy, monoclonal or polyclonal, demonstrated a similar spread in values for both lymphoid populations compared to the "unselect" GD cohort.

The CD4/CD8 ratio was significantly higher ($p<0.001$) in healthy controls (1.8±0.7; mean±SD) compared to that found in GD patients (1.0±0.7). Excluding patients with undetectable chitotriosidase, as this is likely to represent those with homozygous null mutations and thus not representative of disease activity, and those with unavailable data, there was no correlation between current chitotriosidase activity and the percentage

of CD3+/CD4+ve or CD3+ve/CD8+ve cells (Figure 5-13). Limitations of this analysis include the low number of patients, absent documentation of the chitotriosidase genotype (null mutations) and the fact that the majority of patients were on ERT with plasma chitotriosidase activities less than 2000nmol/hr/ml. Pre-ERT chitotriosidase activity did not correlate with the current percentage of CD3+ve/CD4+cells (r^2=0.004, p=0.76) or CD3+ve/CD8+ve cells (r^2=0.006, p=0.73).

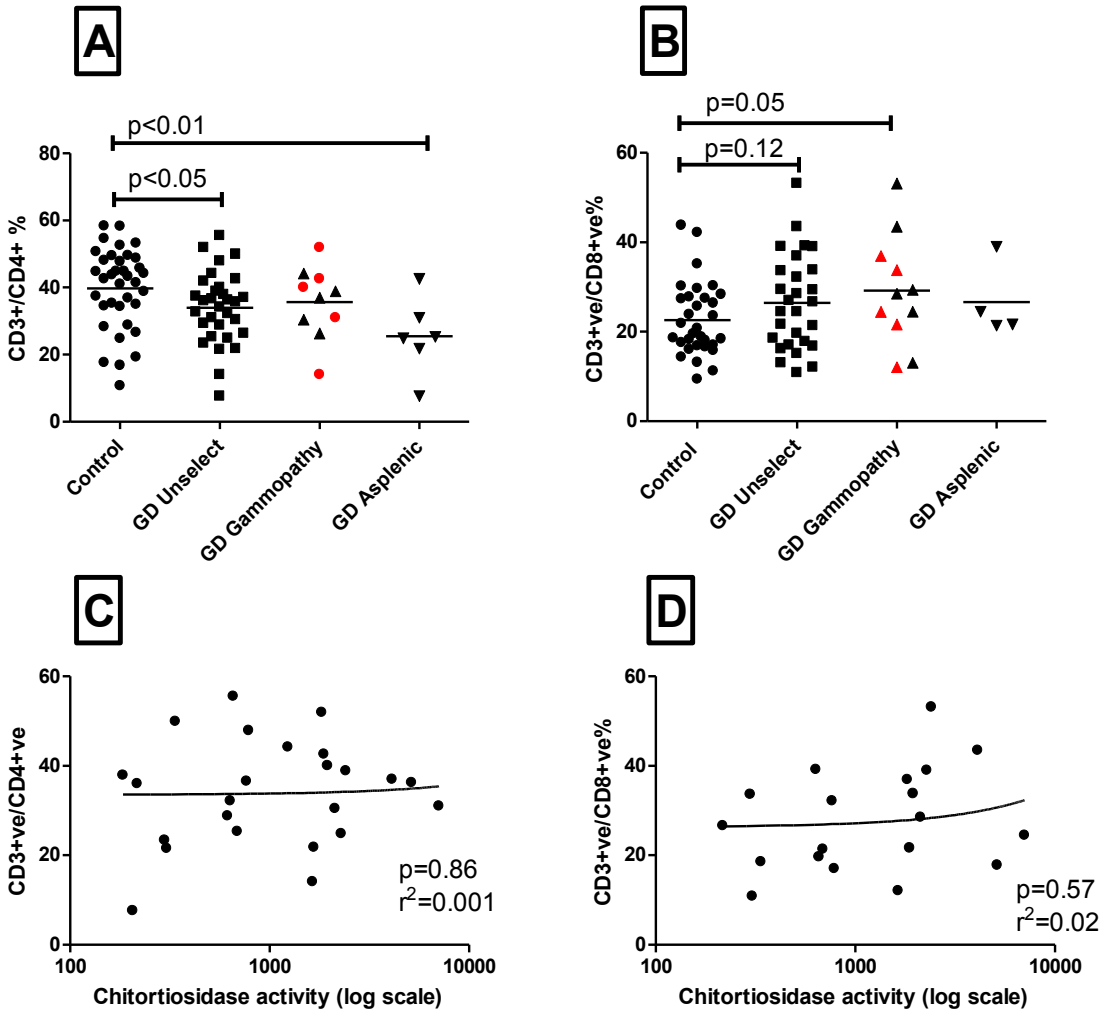


Figure 5-13. Quantification of CD4+ve and CD8+ve peripheral T-cells in control and GD patients. Percentage of (A)CD3+ve/CD4+ve and (B)CD3+ve/CD8+ve cells. GD patients with a paraprotein are represented in red within the GD gammopathy cohort. Mean indicated by the horizontal line. Log chitotriosidase activity compared to the % of (C) CD3+ve/CD4+ve and (D) CD3+ve/CD8+ve cells in GD patients.

5.4.4.3 NATURAL KILLER CELL POPULATIONS

Two different subsets of CD56+ve NK-cells were analysed (CD3-ve and CD3+ve NK-cells).

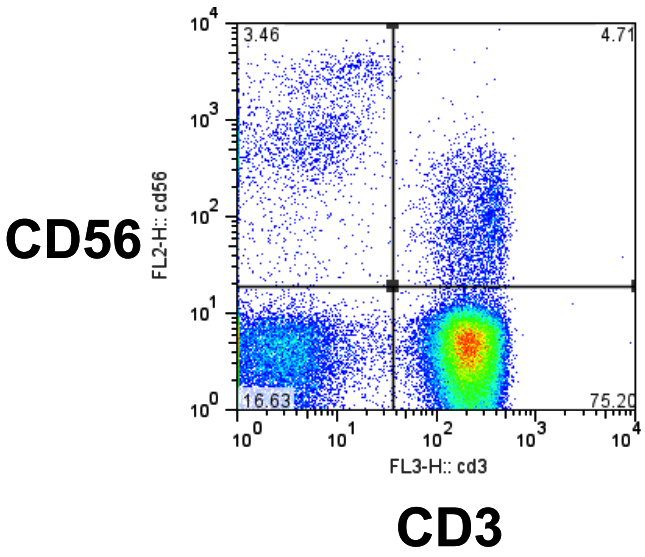


Figure 5-14. Representative flow cytometry plot of lymphocytes using CD3/56 immunostaining.

In comparison to CD3+ve NK-cells, CD3-ve NK-cells expressed CD56 more intensely. The peripheral blood of GD patients contained a higher percentage of CD3+ve NK-cells, but a lower percentage of CD3-ve NK-cells (Table 5-3).

Cells	Control	Gaucher Disease
CD3+ve/CD56+ve %	5.1±3.1	9.5±7.6**
NK CD3-ve/CD56+ve %	12.9±7.7	9.0±4.7*
CD56+ve %	18.1±7.7	19±8.8

Table 5-3. NK subsets in control and GD patients. Mean±SD. *p<0.05 **p<0.01

Compared to the unselected GD population, patients with gammopathy, including those with an M-band, had a similar mean and spread of results for both NK populations. Asplenic patients had an identical distribution of results to GD patients with gammopathy and the unselected GD cohort. In patients with GD, current chitotriosidase activity did not correlate with the percentage of CD3+ve/CD56+ve (r^2=0.1, p=0.13) or CD3-ve/CD56+ve cells (r^2=0.1, p=0.14). Pre-ERT chitotriosidase activity did not

correlate with the current percentage of CD3-ve/CD56+cells (r^2=0.008, p=0.95) or CD3+ve/CD56+ve cells (r^2=0.0001, p=0.73).

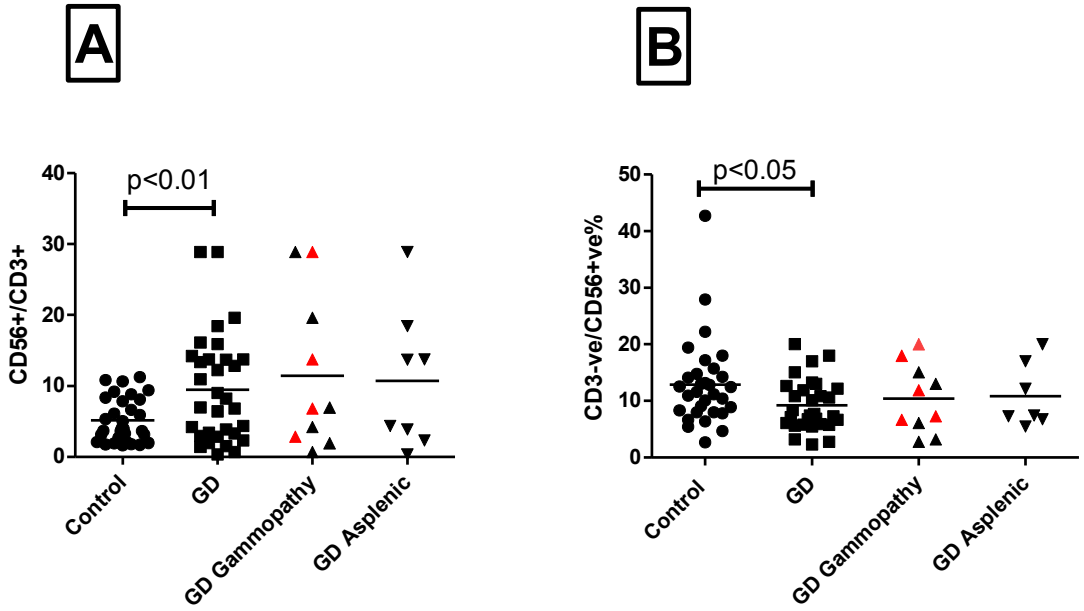


Figure 5-15. Percentage of (A) CD3+ve/CD56+ve and (B) CD3-ve/CD56+ve cells. GD patients with a paraprotein are represented in red within the GD gammopathy cohort. Horizontal line indicates the mean.

5.4.4.4 INVARIANT NK-T CELLS

Invariant NK-T cells (CD3+ve/6B11+ve) are known to expand when presented with sphingolipid on CD1d tetramers. As sphingolipid metabolism is abnormal in GD, it was hypothesised that the number of iNK-T cells may be different to that observed in healthy controls. The number of 6B11+ve iNK-T cells reported below is a ratio to the total number of CD3+ve T-cells. Compared to healthy controls, GD patients had a significantly lower percentage of iNK-T cells (0.14%±0.09 versus 0.07%±0.07; mean±SD; p=0.001; Figure 5-16). Despite low patient numbers in the GD gammopathy group, there was still a highly significant decrease in the percentage of iNK-T (p<0.01) compared to controls. Chitotriosidase activity, pre-ERT (r^2=0.008, p=0.95) or current (r^2=0.04, p=0.51), did not correlated with the percentage of iNK-T cells.

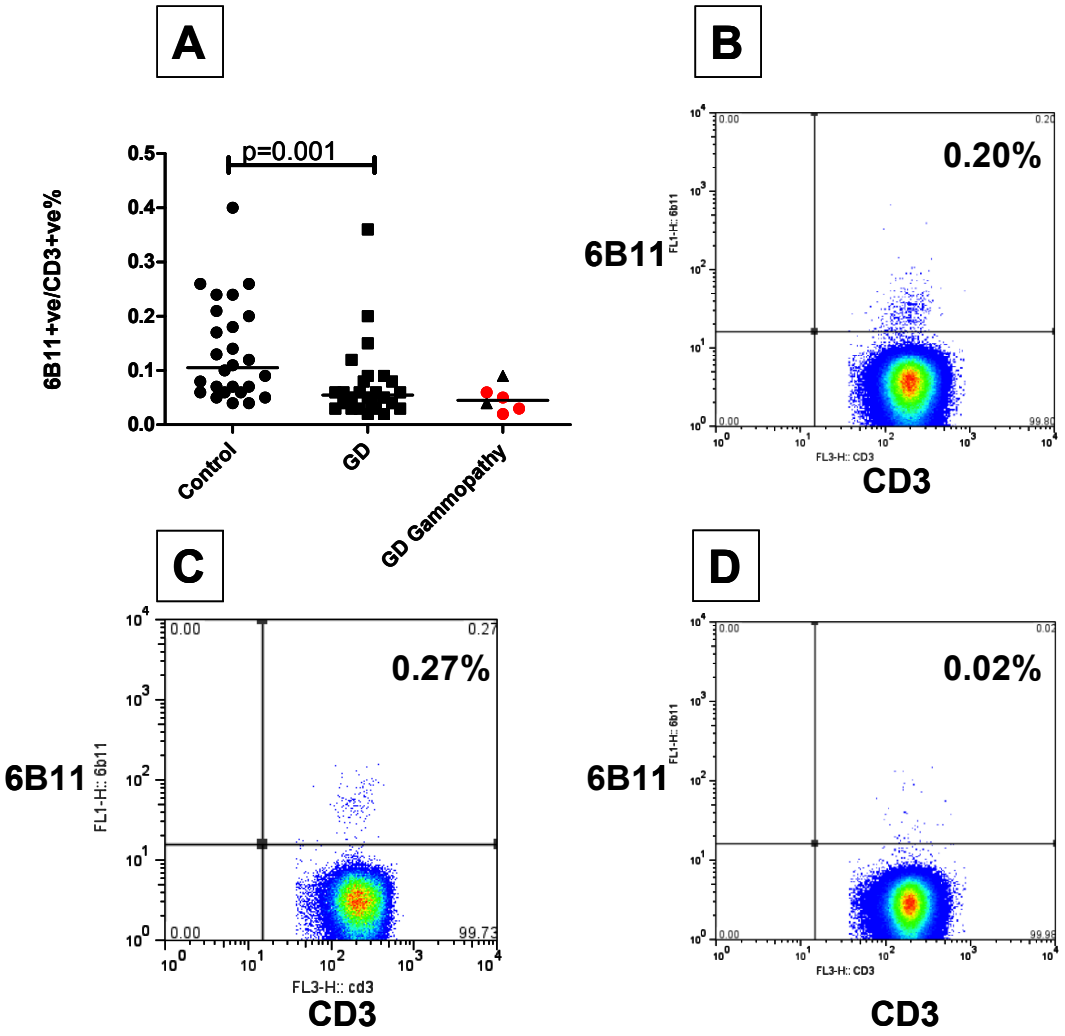


Figure 5-16. (A) Percentage of CD3/6B11+ve invariant NK cells in healthy controls and GD patients. Line represents median. GD patients with a paraprotein are represented in red in the GD gammopathy cohort. Flow cytometry plots from two controls (B, C) and a GD patient (D). Percentage of iNK-T cells is reported in the right upper quadrant.

5.4.4.5 CD1D EXPRESSION

CD1d molecules are expressed on antigen presenting cells, including peripheral blood monocytes. The MFI of CD1d was statistically higher on GD monocytes compared to monocytes derived from healthy controls (78.1±18.1 versus 99.0±17.7; mean±SD).

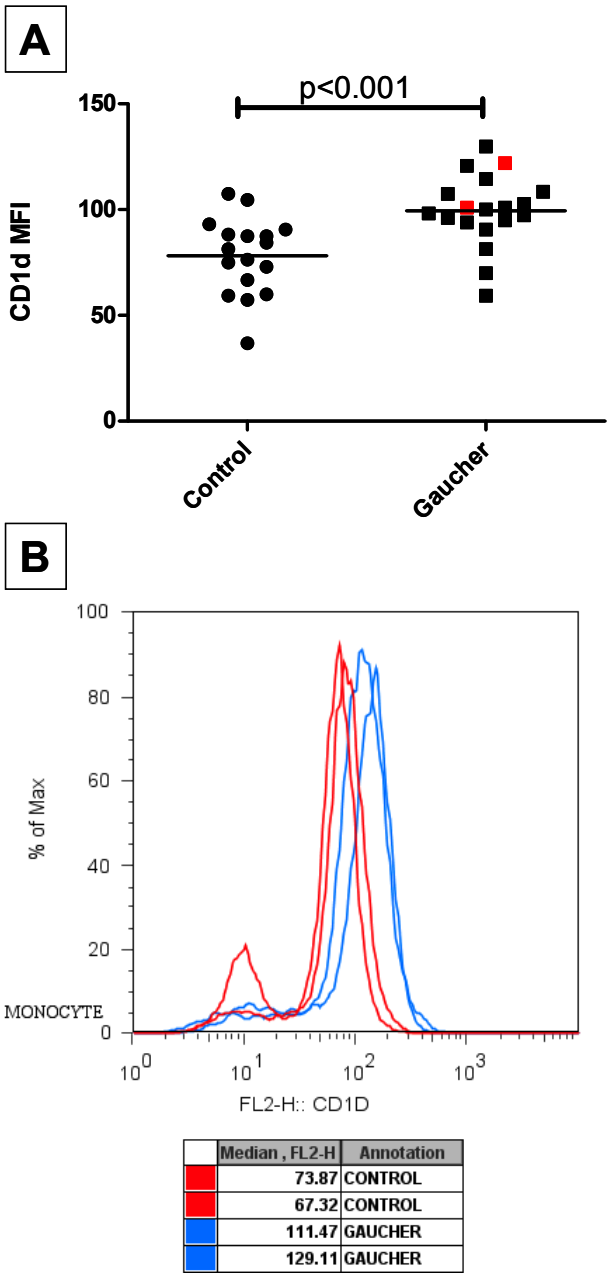


Figure 5-17. (A) Median fluorescent intensity of CD1d molecules on monocytes derived from healthy controls and GD patients.
Mean represented by horizontal line. GD patients with a paraprotein are represented in red. (B) CD1d histogram representing two patients with GD (red) and two healthy controls (Blue).

The GD patient with the highest MFI was a newly diagnosed patient who was treatment-naïve (CD1d MFI = 129). Excluding patients with absent chitotriosidase activity, there was no correlation between CD1d expression and plasma activity (r^2=0.04, p=0.5). Furthermore, pre-ERT chitotriosidase activity did not correlate with the MFI of CD1d (r^2=0.14, p=0.13).

5.4.5 LIPID STIMULATION EXPERIMENTS

Results reported above that patients with GD have a lower percentage of iNK-T cells and elevated expression of CD1d on peripheral blood monocytes. As discussed in section 5.2.2, iGB3 and αGalCer are examples of glycolipids that can stimulate and expand iNK-T cells when presented by APCs on CD1d tetramers. As the percentage of peripheral iNK-T cells is low in GD, it was hypothesised that these cells may have a blunted response to glycolipid stimulation. PBMCs were cultured for 14 days in the presence of IL-2 (200iu/ml) alone, IL-2 plus iGB3 (100ng/ml) or IL2 plus αGalCer (100ng/ml). On D1 and D14 the percentage of iNK-T cells was determined. This assay was used to determine the ability of GD monocytes to present glycolipid and expand iNK-T cell numbers.

5.4.5.1 MONOCYTE QUANTIFICATION:- PRE-CULTURE

Patients with GD had an identical percentage of peripheral blood monocytes (CD14+ve/CD64+ve) compared to healthy controls (Controls 14.4%±5.1 versus GD 14.7%±5.5; mean±SD).

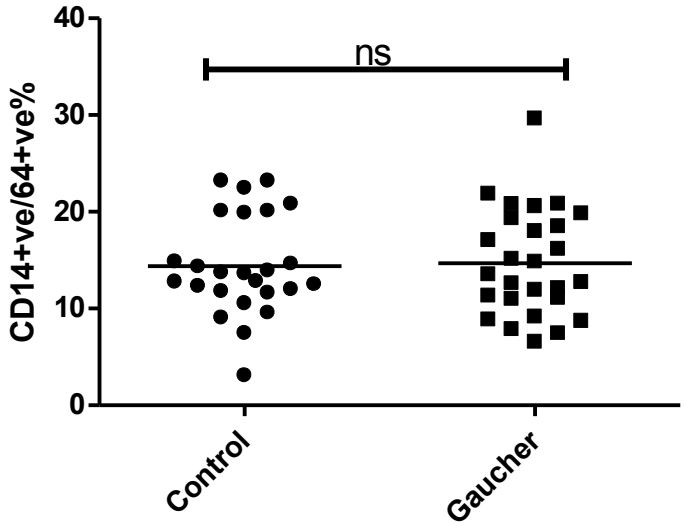


Figure 5-18. Percentage of CD14/64 monocytes in healthy controls and GD patients. Mean indicated by horizontal line.

Therefore, it was assumed in the subsequent lipid stimulation experiments that GD and controls had an equal number of monocytes capable of CD1d presentation in culture. It was shown above that GD monocytes have a higher surface expression of CD1d than cells derived from healthy controls.

5.4.5.2 LIPID STIMULATION EXPERIMENTS – ABSOLUTE INVARIANT NK-T PERCENTAGE

The percentage of CD3+ve/6B11+ve cells, pre-culture, was higher in the PBMCs of healthy controls (0.08±0.04; mean±SD; n=12) compared to those with GD (0.06±0.03; n=9; $p<0.05$). Bi-polar microscopy at D7 revealed a vast increase in cell number and the formation of lymphocyte proliferation centres. Two week culture led to an increase in the percentage of CD3+ve/6B11+ve cells from both GD patients ($p<0.01$) and controls ($p<0.01$). Following 14 days of culture in αGalCer supplemented wells, there was a higher percentage of iNK-T cells in experiments derived from controls compared to those from GD patients ($p<0.05$). There was no difference in the percentage of iNK-T cells harvested from GD or control wells supplemented with IL-2 alone ($p=0.43$) or IL-2 plus iGB3 ($p=0.27$). Paired experiments derived from controls, supplemented with αGalCer, had a higher number of iNK-cells compared to IL-2 culture alone ($p=0.001$; Figure 5-19). The addition of glycolipid did not lead to an increase in iNK-T cells in paired experiments from GD patients (Figure 5-20).

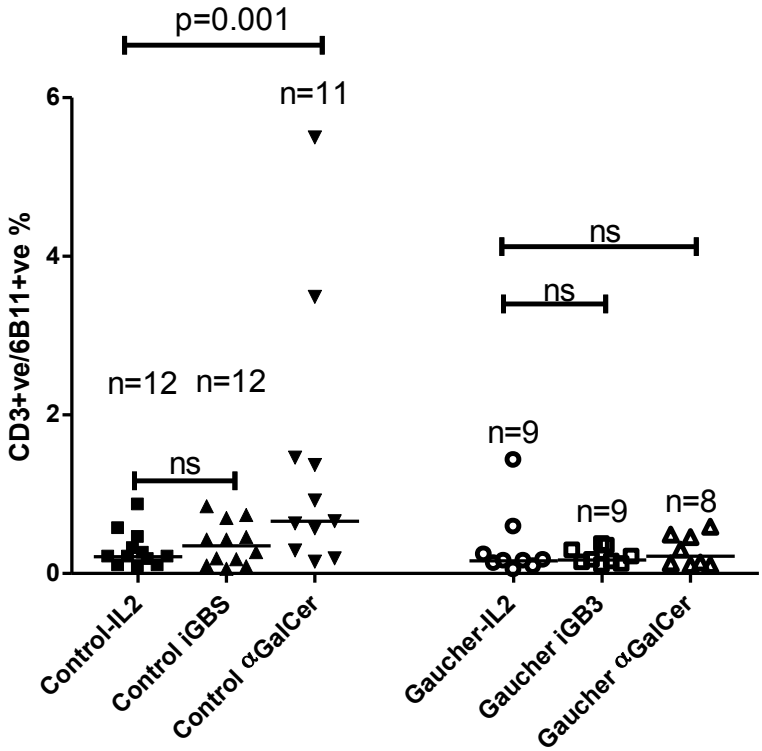


Figure 5-19. Absolute percentage of invariant NK-T cells following 14 day culture (control or GD derived), in IL2 alone, iGB3 or αGalCer. Horizontal line indicates group median. Statistical analysis between groups was performed using a Wilcoxon signed rank test.

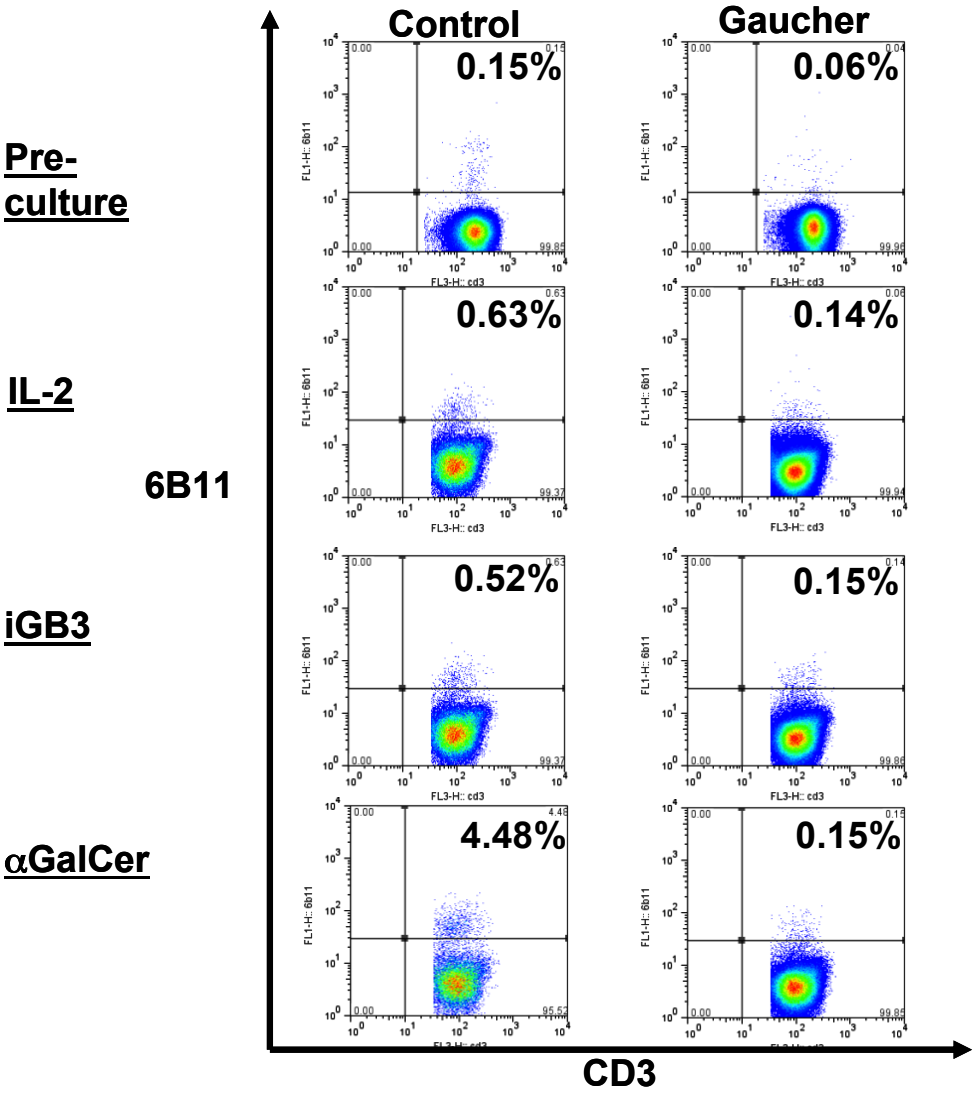


Figure 5-20. Representative flow cytometry plots of invariant NK-T cells on D14 (control or GD derived), in IL2 alone, iGB3 or αGalCer.
The epitope 6B11 is specific for the CDR3 region on the invariant α-chain.

5.4.5.3 INVARIANT NK-T EXPANSION – POPULATION DOUBLING

Population doubling (PopD) was calculated as PopD = log(D14/D0)/log2 where D14 and D0 represent the percentage of CD3+ve/6B11+ve cells. Paired PBMC cultures, derived from healthy controls, led to a significantly shorter population doubling time in the presence αGalCer (p=0.001). Analysis between all other groups, either control or GD-derived, did not convey a proliferative advantage over wells grown in IL-2 alone.

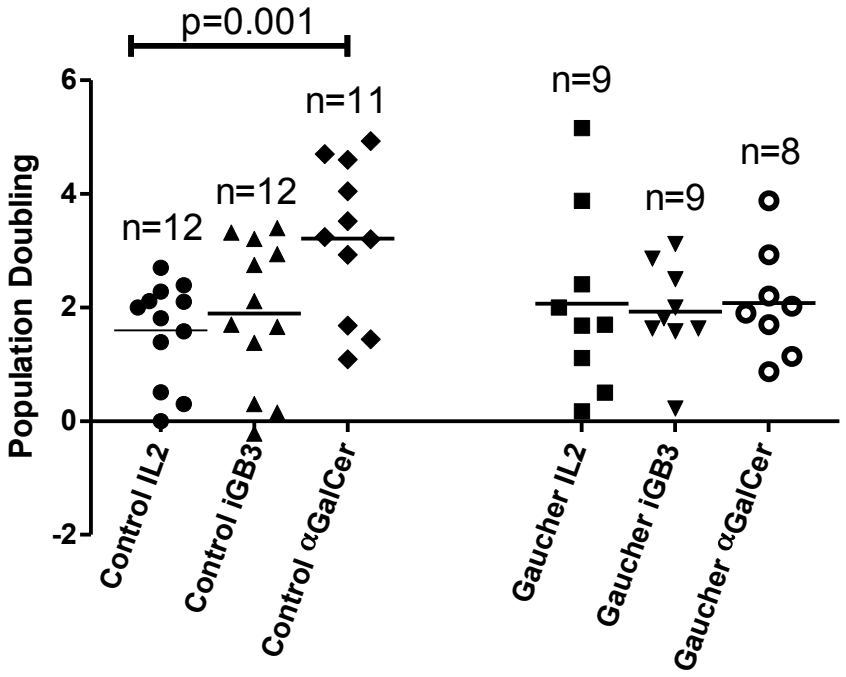


Figure 5-21. Population doubling of iNK-T cells on D14 (control or GD derived) in IL2 alone, iGB3 or αGalCer.
Mean indicated by horizontal line. Statistical analysis between groups was performed using a Wilcoxon signed rank test.

5.4.5.4 INVARIANT NK-T EXPANSION – CBE TREATMENT

As demonstrated in the section above, αGalCer was unable to expand iNK-T cells in GD-derived cultures. It was hypothesised that the percentage of iNK-T cells in culture could be attenuated in αGalCer stimulated wells by the addition of CBE, an inhibitor of lysosomal glucocerebrosidase. PBMCs were incubated, in duplicate ($1x10^6$/mls), in the presence of IL-2 and several different concentrations of CBE for 8 days. On D8, glucocerebrosidase activity was determined and expressed as a ratio of the total protein content. Nearly all CBE concentrations tested led to a 50% reduction in glucocerebrosidase activity (Figure 5-22). In addition, control PBMCs were cultured in a 96-well plate (in triplicate) and exposed to several different concentrations of CBE for 7 days before undergoing MTS dye reduction assay (2 hour). CBE was not toxic to PBMCs (n=3). Based on these results, 50μM and 100μM CBE was used in subsequent experiments. These results are consistent with those reported by other investigators in which primary cells were cultured with CBE concentrations between 50-500 μM[427;488].

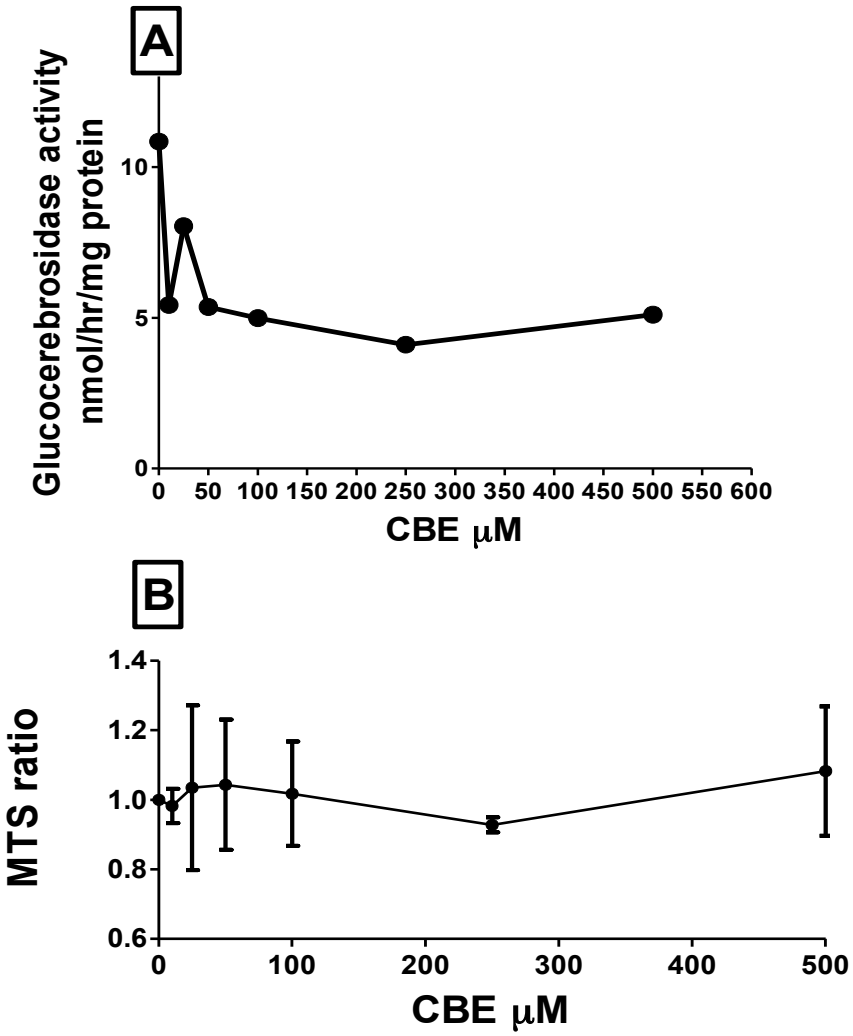


Figure 5-22. (A) Glucocerebrosidase activity of D8 PBMC cultures (controls) grown from D1 in several different concentrations of CBE (derived from duplicate cultures). (B) MTS toxicity assay on D7 PBMC cultures (control) subjected to CBE (n=3).
Mean and standard deviation plotted.

PBMCs from two healthy controls (previously noted to expand their iNK-T cell population following stimulation with αGalCer) were grown in αGalCer, αGalCer plus 50μM CBE or αGalCer plus 100μM CBE for 10 days. CBE at both concentrations led to a reduction in the percentage of D10 iNK-T cells (Figure 5-23).

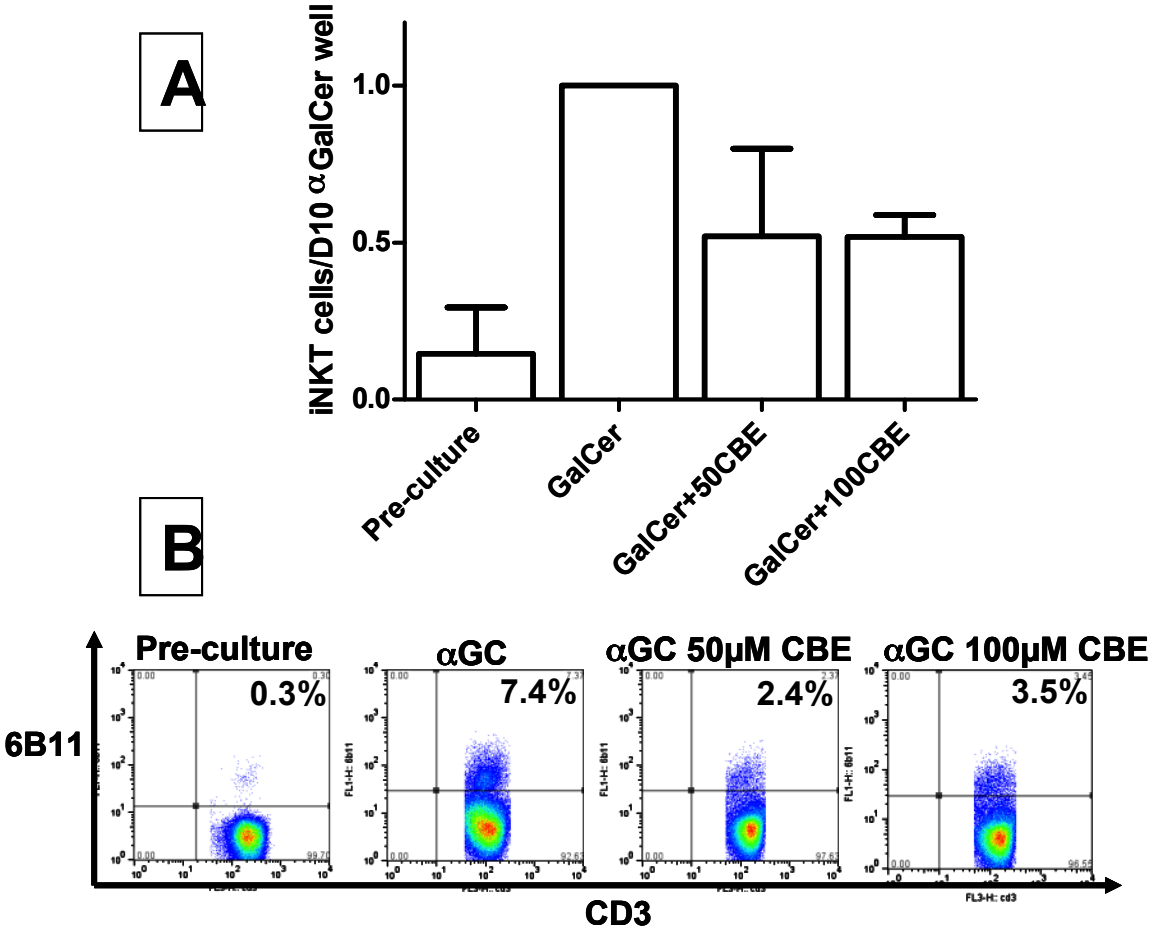


Figure 5-23. Invariant NK-T lipid stimulation assays in the presence of CBE.
(A) Results of two individual experiments following 10 day culture with αGalCer alone or, in addition, CBE (50μM, 100μM). Results reported as a ratio to the percentage of iNK-T cells harvested from the paired D10 αGalCer well. Mean+SD plotted (n=2). (B) Flow cytometry plots represent the results acquired from experiment A. Number of iNK-T cells represented in the upper right quadrant.

5.4.6 NK-CELL AND PBMC KILLING ASSAYS

The results reported here demonstrate both numerical abnormalities within individual T-cell subsets and the inability of αGalCer to expand GD iNK-T cells. Immune cells derived from GD patients (effector cells) maybe less able to kill tumour cells (target cells) due to impaired lysosomal function. Killing assays were performed on three different immortalised cell lines (NCI-H929, K562 and JJN3) with unstimulated NK-cells and IL-2 primed cells (NK or PBMCs). NK-cells were isolated from the buffy coat of peripheral blood based on CD56 expression using MACs column technology.

5.4.6.1 VALIDATION OF MODEL; IL-2 ENHANCES NK-CELL KILLING

Control-derived NK-cells (n=4) were cultured overnight, in paired experiments, in the presence or absence of IL-2 (200iu/ml). Killing assays were performed the following day with a NK (effector cell) to target ratio of 5:1. Target cells were pre-labelled with the intracellular dye PKH26 and the percentage of TO-PRO+ve cells (apoptotic) was determined 4 hours post co-culture. NK-cells, stimulated with IL-2, demonstrated an increased ability to kill immortalised cell lines compared to IL-2 naïve cells (Figure 5-24).

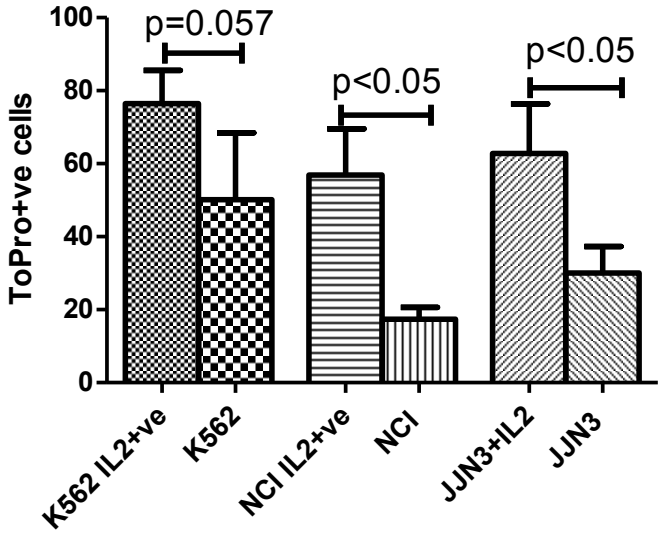


Figure 5-24. IL-2 enhanced the killing of cell lines by NK-cells derived from healthy controls (n=4). Mean+SD plotted.

5.4.6.2 VALIDATION OF MODEL:- NK CELLS MEDIATE THE MAJORITY OF PBMC CYTOTOXICITY

Killing assays were performed on control CD56+ve and CD56-ve cells, as described above, with an effector:target ratio of 5:1 (n=4). The CD56-ve fraction contains cytotoxic T-cells, helper T-cells, B-cells and monocytes. Figure 5.25 shows that CD56-ve cells inflict relatively little target cell death compared to the CD56+ve fraction.

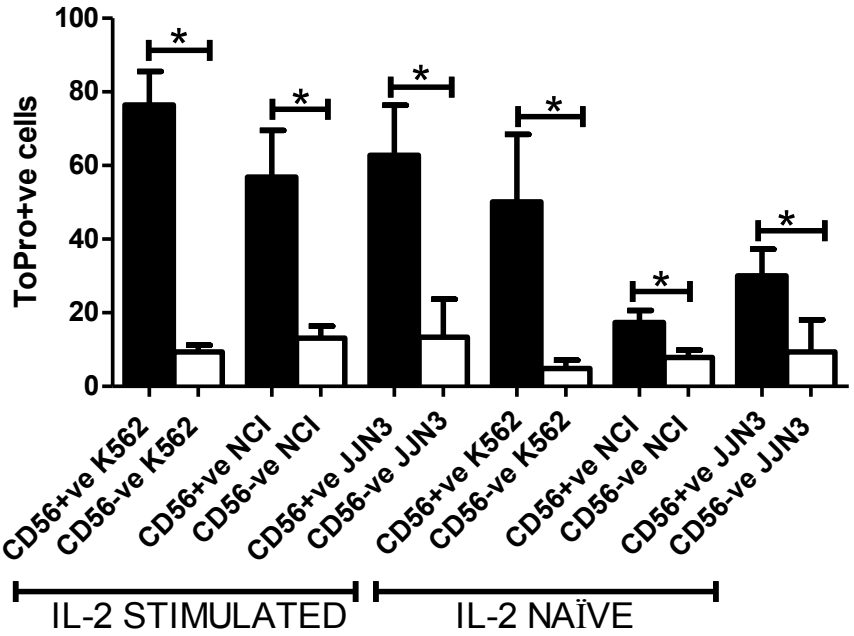


Figure 5-25. The CD56-ve fraction compared to the CD56+ve fraction harbours comparatively little cytotoxic activity (n=4). Percentage of TO-PRO+ve cells reported.
This comparison holds true for both IL-2 stimulated and IL-2 naïve cells. Mean+SD plotted.*p<0.05

5.4.6.3 CONTROL VERSUS GAUCHER NK KILLING ASSAYS

Paired experiments were performed comparing aged-matched healthy controls and GD patients with an effector:target ratio of 5:1. The results are shown in Table 5-4.

	Gaucher:Control TO-PRO+ve (Mean±SD)
K562 IL2 (n=4)	1.1±0.12
K562 (n=4)	1.2±0.2
NCI-H929 IL2 (n=4)	1.0±0.2
NCI-H929 (n=4)	1.3±0.6
JJN3 IL2 (n=4)	1.0±0.15
JJN3 (n=4)	1.2±0.5

Table 5-4. Killing assay reported as a ratio of killed target cells in GD assays divided by the percentage in control assays. Mean±SD reported (n=4).

Greater cell kill was observed in K562 cells, irrespective of IL-2 supplementation, compared to the NCI-H929 and JJN3 cells lines. NK-cells derived from patients with GD, compared with NK-cells derived from healthy controls, were equally effective in inducing cell death in the 3 target cell lines in response to IL-2 stimulation (Figure 5-26;

Figure 5.27). IL2-naïve NK-cells derived from patients with GD led to an equivalent number of apoptotic cells in the 3 target cell lines. In fact, un-stimulated CD56+ve cells from controls demonstrated an inferior ability to induce cell death in the K562 cell line in 3 out of 4 paired experiments. The GD patient reported in experiment A exhibited the highest level of killing from IL2-naïve NK cells in all three cell lines. The overnight stimulation of NK-cells with IL-2 led to a higher percentage of apoptotic cells in GD experiments, but statistical significance was only reached for the NCI-H929 cell line ($p<0.05$; MW). This may be due to insufficient patient numbers. None of the patients with GD in the tested cohort had an M-band. It was therefore concluded that GD-derived NK-cells are not inferior to controls, based on this *in vitro* killing assay.

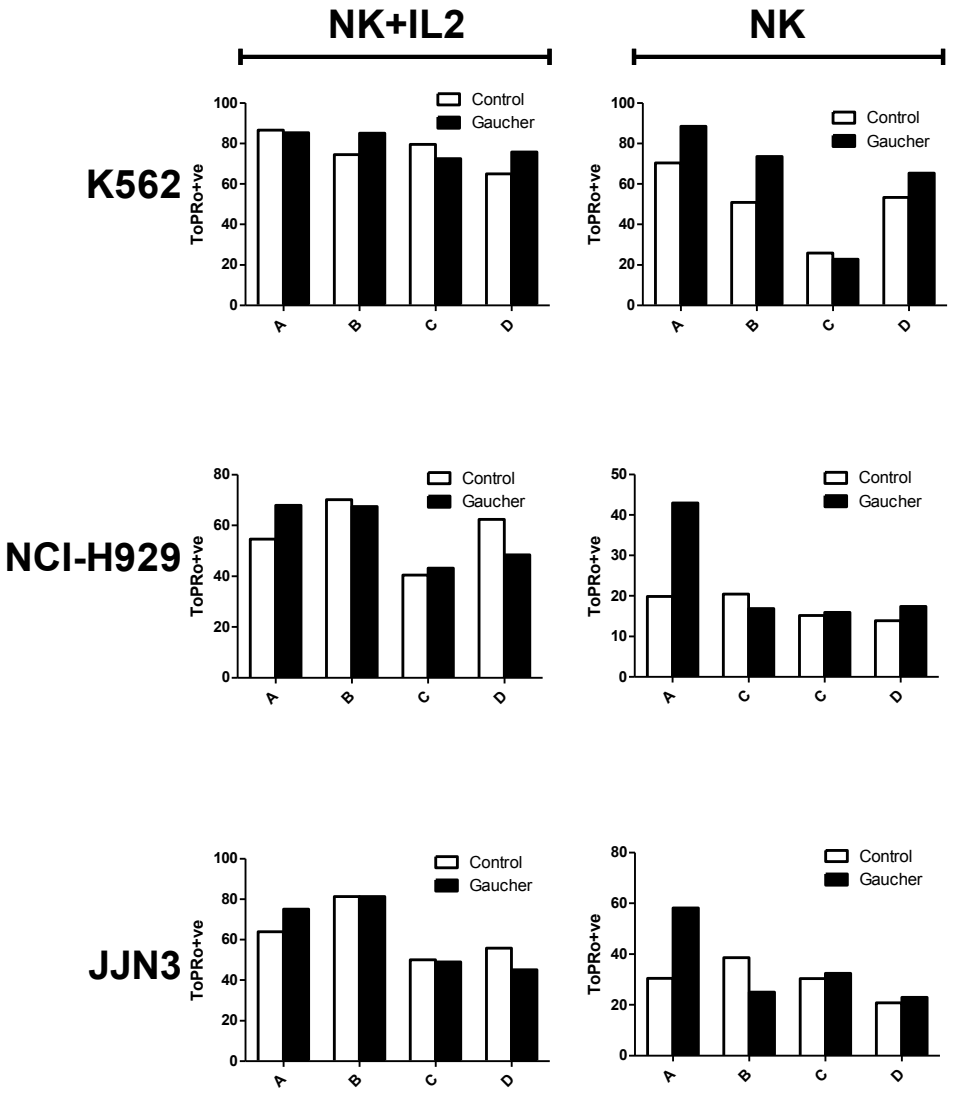
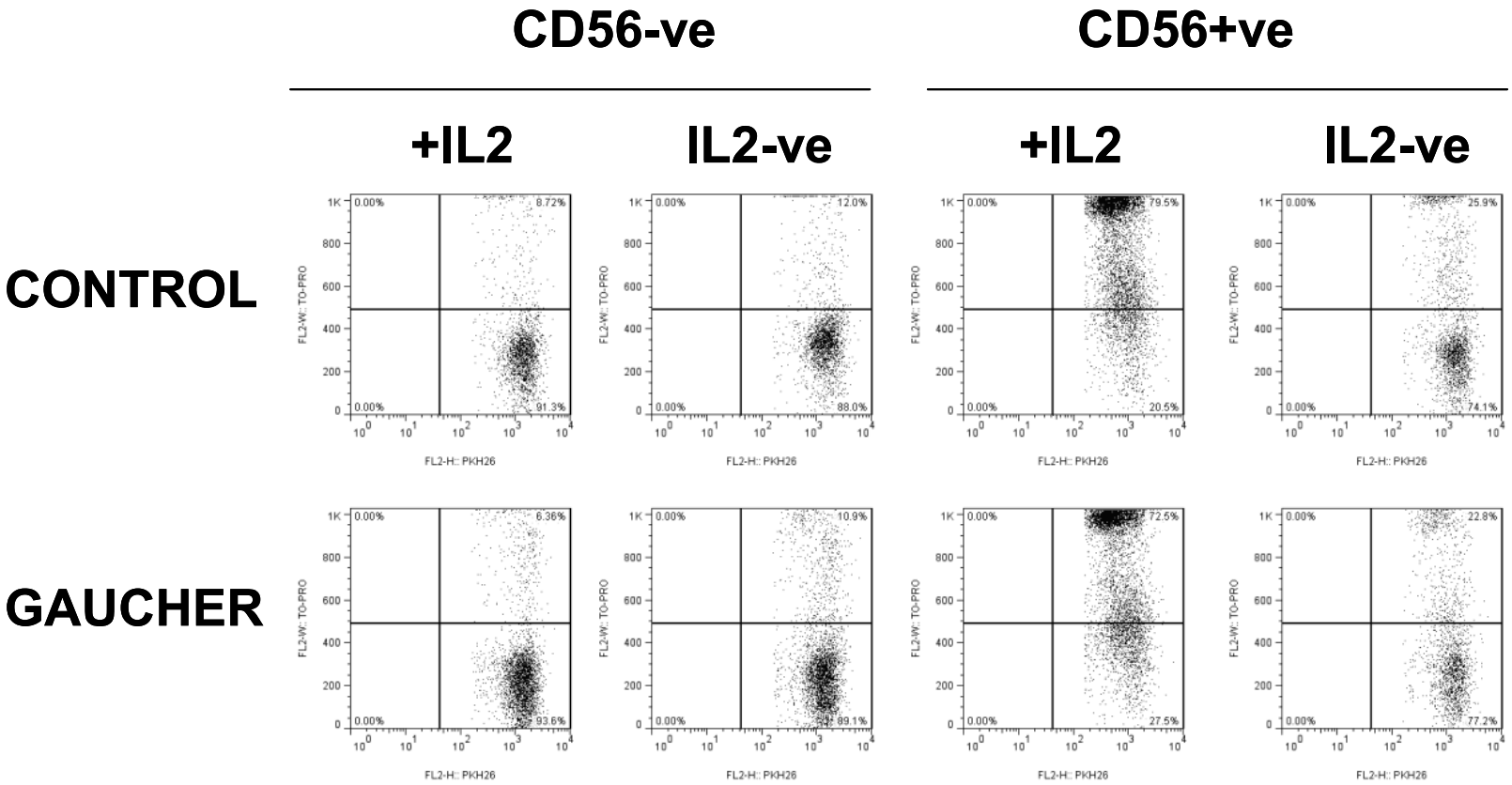


Figure 5-26. NK-killing assay comparing age matched control and GD patients (n=4; A-D).

Figure 5.27. NK Killing assay. Representative flow cytometry plots. K562 cells were pre-labelled with PKH26 and the percentage of To-PRO+ve cells post killing assay is represented in the upper right quadrant



5.4.6.4 CONTROL VERSUS GAUCHER CD56-VE KILLING ASSAY

Compared to healthy controls, IL2-naïve CD56-ve cells from GD patients were able to induce more cell death in NCI-H929 cells compared to cells from healthy controls ($p<0.05$). IL-2 stimulation of the CD56-ve fraction derived from control cultures led to an increase in the percentage of apoptotic cells in both the K562 ($p<0.05$) and NCI-H929 ($p=0.057$) cell lines, confirming the cytotoxic capacity of this fraction. This was similar to that seen in GD killing assays where the addition of IL-2 led to a higher percentage of apoptotic cells in the K562 cell line ($p<0.01$), but this failed to reach significance in the NCI-H929 cell line ($p=0.09$). Therefore, it was not possible to demonstrate inferior killing in the non-NK fraction from GD patients when compared to healthy controls.

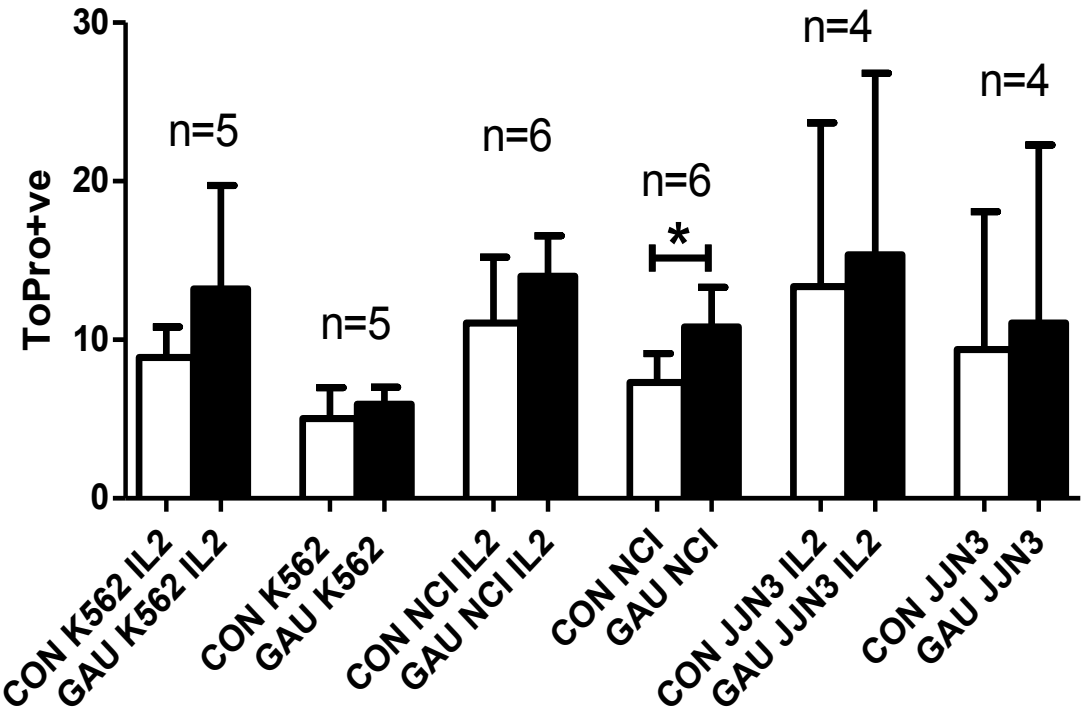


Figure 5-28. Percentage of TO-PRO+ve cells post 4 hour killing assay with the CD56-ve fraction from controls and Gaucher patients. Mean+SD plotted. *p<0.05

5.4.6.5 CONTROL VERSUS GAUCHER PBMC KILLING ASSAYS

As demonstrated above, GD patients have decreased CD4, iNK-T and CD3-ve NK-cell numbers, in addition to an increased percentage of CD3+ve NK cells. Therefore, PBMC killing assays were performed to replicate not only these numerical differences but to exploit any functional abnormalities that may exist in the interactions of GD blood cells. PBMC killing assays, pre-stimulated with IL-2 overnight, were performed with K562, JJN3 and NCI-H929 cells at effector:target ratios of 40:1, 20:1 and 10:1.

Compared to healthy controls, cultures from patients with GD demonstrated an impaired ability to kill K562 cells at all effector:target ratio (Table 5-5; Figure 5-29; Figure 5-30). The mean percentage of kill in K562 killing assays decreased in parallel with the reduction in effector:target ratio.

	Control TO-PRO+ve Mean±SD (n=12)	**Gaucher TO-PRO+ve Mean±SD (n=12)**	**Paired t-Test**
K562 40:1	56.3±12.1	45.1±15.2	<0.01
K562 20:1	48.9±16.6	36.1±13.2	<0.05
K562 10:1	37.3±18.2	26.2±11.9	<0.05
NCI 40:1	9.4±8.3	12.5±8.8	NS
NCI 20:1	9.7±6.8	10.4±8.1	NS
NCI 10:1	18.9±6.9	7.6±6.2	<0.0001
JJN3 40:1	16.9±10.5	18.11±9.4	NS
JJN3 20:1	14.9±9.9	13.6±8.4	NS
JJN3 10:1	9.1±6	9.2±6.7	NS

Table 5-5. Percentage of apoptotic To-Pro+ cells in killing assays performed in K562, NCI-H929 and JJN3 cells cultured with PBMCs-derived from controls or GD patients. NS = non-significant.

The mean percentage of apoptotic cells above background cell death was less than 20% at all concentrations for both myeloma cell lines. Curiously, control PBMCs were more effective at killing NCI-H929 cells at a ratio of 10:1 than 20:1 ($p<0.01$; paired t-test).

The percentage of TO-PRO+ve NCI-H929 cells was higher in control killing assays compared to GD-derived cells at the same effector concentration of 10:1 ($p<0.0001$).

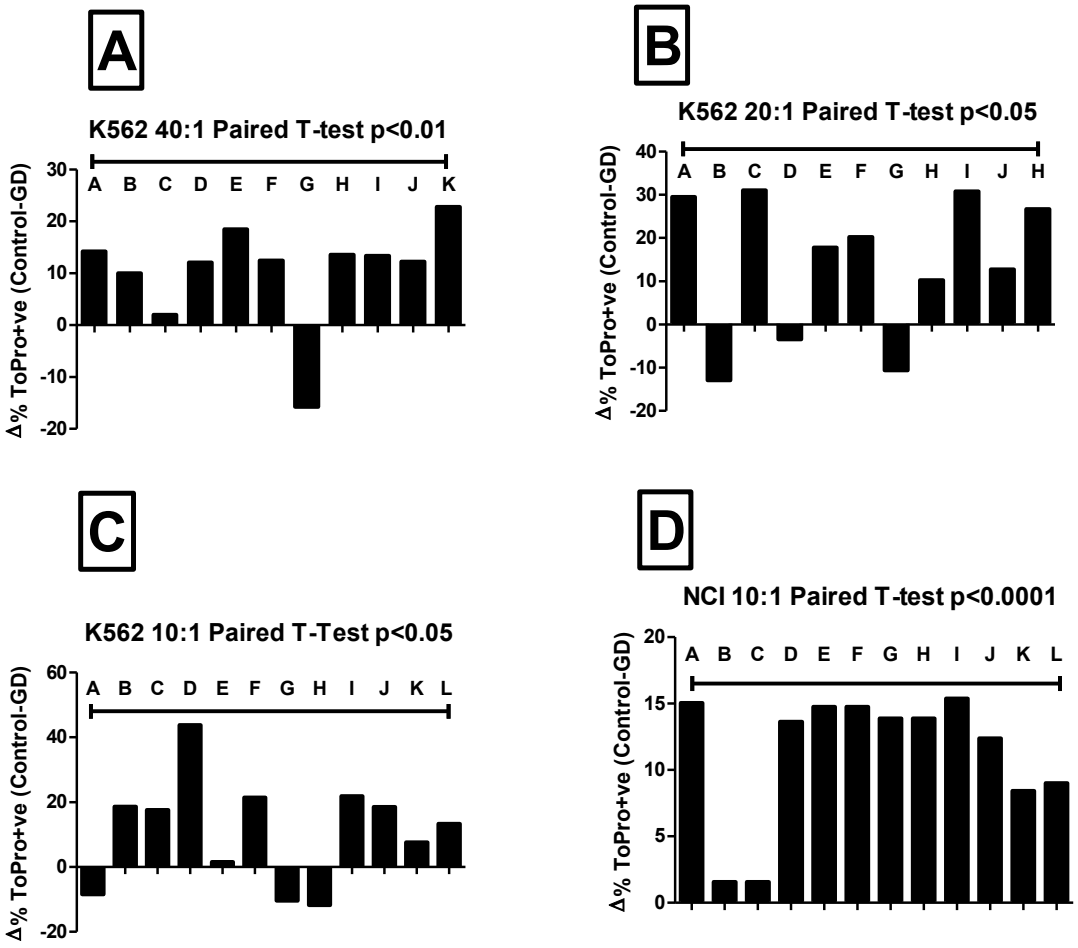
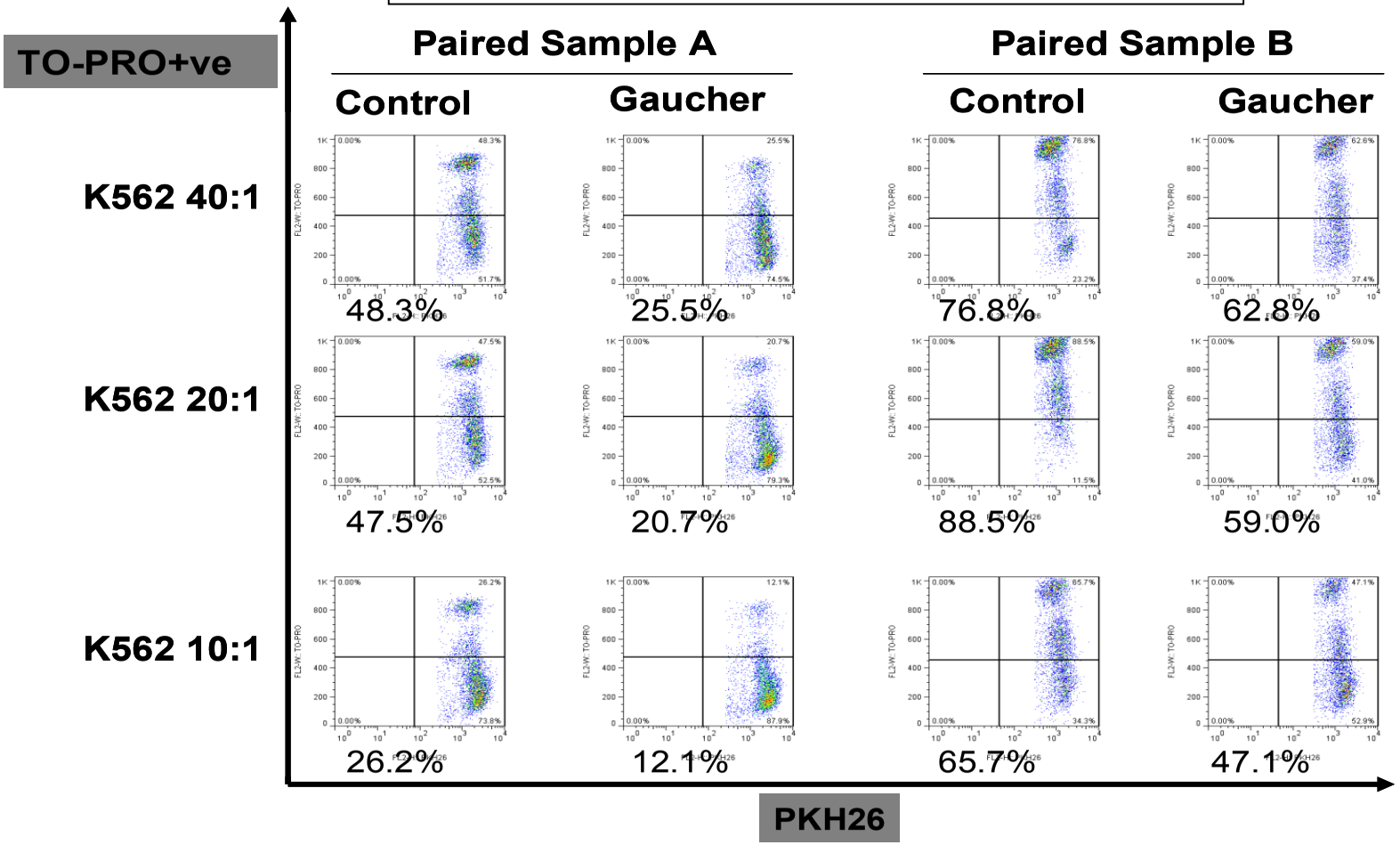


Figure 5-29. Graphical representation of paired killing assays (n=12) documenting the numerical difference in TO-PRO+ve cells between controls and GD patients.
Each letter represents a temporally paired experiment, control versus GD (A-L).

Figure 5.30. Representative flow cytometry plots of PBMC cytotoxicity assays comparing control and GD patients. The percentages refer to cells in the right upper quadrant (apoptotic).



5.5 DISCUSSION

The novel results reported in this chapter demonstrate abnormalities within the lymphoid fraction of GD patients that could confer impaired tumour surveillance. Compared to healthy controls, patients with GD had a higher percentage of CD3+ve NK-cells, a decreased CD4:CD8 ratio and a reduction in the percentage of CD3-ve NK, iNK-T and CD4+ve T-cells. The results also show that GD lymphocyte subsets have depleted glucocerebrosidase activity. Lysosomal dysfunction was confirmed in GD lymphocytes on the basis of abnormal intra-cellular trafficking of lactosylceramide. Compared to healthy controls, GD patients demonstrated an inability to expand iNK-T cells in culture with αGalCer supplementation. GD monocytes had abnormally high cell surface CD1d expression. PBMCs, generated from patients with GD, displayed an inferior ability to kill co-cultured immortalised cell lines. However, no difference was demonstrated in the ability of NK-cells, derived from healthy controls and those with GD, to kill K562, NCI-H929 or NCI-H929 cells.

The following evidence supports the immunological importance of lymphocytes in tumour control. A low ALC predicts for an inferior outcome in several haematological malignancies, including follicular lymphoma, DLBCL and Hodgkin lymphoma[467]. In addition, patients with myeloma who have a low ALC have an inferior response to treatment with bortezomib and dexamethasone[489]. Thus, more aggressive tumours may give rise to a cytokine profile that suppresses lymphocyte production. Successful tumour control has been correlated with an early recovery in the ALC following stem cell transplantation (autograft or allograft)[467]. Marti *et al*. (1988) suggested a high normal ALC in treatment-naïve patients with GD. However, these findings are questionable given the size of the cohort (n=8) and the fact that 4/8 patients had been splenectomised[139]. Splenectomised individuals, irrespective of aetiology, commonly develop a lymphocytosis, which is partly due to an increase in large granular lymphocytes[490].

Numerical differences were not demonstrated in the absolute number or percentage of lymphocytes derived from healthy controls and patients with GD. In addition, the ALC

was identical in GD patients, irrespective of treatment status or the presence of gammopathy (monoclonal or polyclonal). These finding do not support the hypothesis that chronic immune stimulation leads to the development of polyclonal lymphocytosis in GD patients. Inflammatory cytokines, including IL-1β and TNF-α, have been reported to be elevated in the peripheral blood of patients with GD, although these findings have been inconsistent between investigators[73;76;77;81]. Immunocytochemistry on splenectomy specimens failed to demonstrate the expression of a Th1 pro-inflammatory profile in lipid laden macrophages, including the expression of IL-1β and TNF-α[58]. Interestingly, the percentage of peripheral lymphocytes has been shown to be elevated in a cohort of male patients with Fabry disease (11/22 on ERT), irrespective of treatment status[350]. Although the ALC was preserved in GD, abnormalities of the lymphoid profile, that may confer an increased risk of cancer, were investigated next.

In PBMCs from healthy controls, lymphocytes had relatively low glucocerebrosidase activity compared to monocytes, as demonstrated by flow cytometry. Compared to healthy controls, GD lymphocytes had statistically lower enzyme activity, in keeping with results reported by other investigators[53;346;491]. Additionally, these results show a reduction in glucocerebrosidase activity in individual lymphocyte subsets, including CD3-ve NK cells, CD3+ve NK-cells, CD3+ve cells and CD19+ve cells. Reduced lymphocyte activity compared to that measured in monocytes maybe due to differences in lysosomal number and/or cell size. Studies comparing the activity of beta-galactosidase to glucocerebrosidase could be employed to ascertain whether there are differences in lysosomal glucocerebrosidase activity (controlling for lysosomal number) between different cell types including lymphocyte subsets. The majority of GD patients analysed were receiving ERT, suggesting the inability of treatment to significantly affect residual enzyme activity. Lymphocytes, due to their low residual glucocerebrosidase activity, may be particularly sensitive to enzyme depletion if exposed to sufficient substrate. B-cells, derived from control assays, had statistically higher enzyme activity than T-cells and therefore may be more compromised in deficient states. Whether this contributes to the high incidence of B-cell disorders in GD rather than an increased risk of T-cell disorders is speculative. Glucocerebrosidase-deficient CD34+ve cells in culture

have an impaired ability to form erythroid burst forming units, granulocyte colony forming units (CFUs) and monocyte CFUs, but the ability of these cells to proliferative and differentiate lymphocytes was not explored[53].

Ultrastructural examination failed to demonstrate features consistent with glucosylceramide accumulation, including distended lysosomes, vacuole formation, membrane-bound inclusions and tubular arrays, in GD lymphocytes. This contrasts with the findings of others who have reported abnormalities, using electron microscopy, in both peripheral lymphocytes and EBV transformed B-cells from patients with GD[345;346]. Although often quoted in the literature, it is unclear whether Brűck *et al*. (1991), based on their published report, actually identified ultrastructural abnormalities in GD lymphocytes[465]. In keeping with our findings, others have failed to demonstrate inclusions within GD-derived lymphocytes from skin biopsies[492]. Lymphocytes are relatively young blood cells and lysosomal storage is likely to be related to both the age of the cell and the severity of the enzyme deficiency. The fact that two of our patients were on disease modifying therapy and the other untreated individual had recently presented in middle age with mild disease may have reduced the chance of observing ultra-structural abnormalities. The use of immuno-conjugates raised against glucosylceramide may have improved detection sensitivity. Lymphocyte inclusion bodies have been readily identified in other LSDs, including neuronal ceroid-lipofuscinoses (NCL), Mucopolysaccharidosis (MPS), GM gangliosidosis, mucolipidosis (ML), metachromatic leukodystrophy, glycogenosis and Niemann Pick disease[465;493;494]. These disorders have a poorer prognosis than GD and, therefore, sphingolipid deposition may be more prominent. Ultrastructural abnormalities have also been demonstrated in CD4, CD8 and CD56 lymphocyte subsets from patients with MPS, ML and NCL[494]. Macrophages have a comparatively longer life-span than peripheral blood lymphocytes and thus are more likely to accumulate glucosylceramide over time[495]. In addition, as macrophages have significantly higher glucocerebrosidase activity, they may be more susceptible to the deposition of glucosylceramide in deficient states.

Although structural abnormalities within GD lymphocytes were not demonstrated, they were functionally abnormal on the basis of lipid trafficking. Compared to lymphocytes from healthy controls, BODIPY-tagged lactosylceramide accumulated abnormally in the lysosomes of lymphocytes derived from patients with GD rather than being concentrated in the Golgi apparatus and endosomal compartment. This is in keeping with prior reports of enzyme-deficient macrophages and fibroblasts in GD[487]. Physiological sphingolipid metabolism has been shown to be important in controlling cell apoptosis and proliferation[19-21]. Abnormal sphingolipid haemostasis may confer a proliferative advantage on differentiating B-cells or lead to the apoptotic depletion of specific subsets involved in tumour surveillance.

Using a panel of antibodies (CD19, IgD, CD27, CD21, CD38 and IgM) the percentage of naïve B-cells, IgM memory, class switched, CD21 low expressing B-cells, transitional B-cells and plasmablasts in the peripheral blood of healthy controls and patients with GD was compared. This has previously been described in non-GD individuals[496;497]. Patients with GD had a significantly lower percentage of plasmablasts, but otherwise individual populations were identical to that seen in healthy controls. This was somewhat surprising as it was expected that patients with GD would have a higher percentage of CD19+ve/CD38+ve plasmablasts given the elevated incidence of gammopathy. Additionally, two patients had an established M band. The majority of the GD cohort were, however, receiving ERT and had only modest elevations in their chitotriosidase activity (<2000nmol/hr/ml plasma). These findings, therefore, may not be representative of an untreated cohort with severe disease.

Marti *et al*. (1988) reported a three times higher increase in the mean percentage of CD20+ve lymphocytes in an untreated cohort of patients with GD in comparison to a control population[139]. However, within our cohort (n=10) there was an identical percentage of CD19+ve peripheral B-cells in healthy controls and patients with GD. CD19 was used as a pan B-cell marker which, unlike CD20, additionally identifies more primitive cells, including pre-B and pro-B cells. The fact that our GD cohort was composed of individuals mostly on disease modifying therapy is a more plausible

explanation for these conflicting observations. In addition, by documenting only the percentage of peripheral B-lymphocytes, an increase in the absolute number of CD19+ve cells could have been missed. The literature is relatively sparse in detailing peripheral B-cell populations either pre or post ERT. Khalifa *et al.* (2010) reported a decrease in the percentage of peripheral blood CD19+ve cells in 12/16 paediatric patients, 6 of whom were ERT-naïve (European Haematology Association, Abstract Book, 2010)[235]. This report, however, lacked information detailing the control population and whether a pre-existing reference range was used. Although the percentage of peripheral CD19+ve B-cells has been demonstrated to be elevated in both treated and ERT-naïve Fabry patients, there have been no reports describing an increased incidence of gammopathy[350].

Decreased peripheral T-cells have been reported to impair tumour surveillance[486;498]. In patients with CLL, a higher T-cell/leukaemic monoclonal B-cell ratio has been reported to predict for early stage disease and mutated IgH genes, both associated with a better prognosis[499]. However, neither of these variables was associated with the absolute number of CD3+ve T-cells. In the results reported here, a similar percentage of CD3 cells were found in healthy controls and individuals with GD. This contrasts with the results of other investigators who have demonstrated a decrease in CD3+ve T-cells in a paediatric population, approximately a third of whom were on ERT[235]. In comparison, the majority of GD patients reported here were receiving ERT and speculatively the analysis of untreated individuals may have yielded a different result. Prior data have suggested that ERT leads to the correction of some, but not all, immune defects in GD. Balreira et al. (2005) showed disturbances within CD1d expression, MHC class II expression and the percentage of CD4 plus CD8 lymphocytes in ERT treated individuals. The intensity of MHC class II expression was suggested to fall with treatment. In a cohort of patients with Fabry disease, the mean percentage of CD3+ve T-cells was identical to a control population, irrespective of disease modifying therapy. However, these patients had a mild disease phenotype without evidence of renal failure, cerebrovascular disease or heart failure[350].

The results reported here show that patients with GD have a lower percentage of peripheral CD4+ve T-cells, in addition to a vastly reduced CD4:CD8 ratio when compared to healthy controls. The percentage of CD8+ve cells did not differ between controls and GD patients. However, there was borderline significance towards a higher percentage of CD8+cells in GD patients with gammopathy compared to controls ($p=0.05$). Other investigators have demonstrated a similar decease in the percentage of CD4+ve T-cells, irrespective of treatment, in addition to an increase in the proportion of CD8+ve lymphoid cells in GD patients on ERT only[4]. In keeping with our results, the CD4/CD8 ratio was inverted in 10/16 paediatric patients of whom 10/16 were receiving ERT[235]. Published reports in ERT-naïve individuals have been inconsistent with studies reporting both increases and decreases in the number of CD4 and CD8 +ve T-cells[139;347]. The percentage of CD8+ve T-cells has been shown to be reduced in a cohort of untreated Fabry patients, although the percentage of peripheral CD4+ve cells was identical to that of healthy controls[350]. Fabry disease is caused by a deficiency in α-galactosidase A which results in the accumulation of globotriaosylceramide and diagalactosylceramide plus secondary substrates such as globoside[15]. In comparison to GD, Fabry disease may lead to attenuated glucosylceramide accumulation due to impaired globoside metabolism and the generation of ceramide for glycosylation (glucosylceramide synthase). Therefore, differences in the lipid profile between individual sphingolipidosis may confer a tendency towards certain lymphoid profiles.

With regards to malignancy, anomalies of peripheral blood lymphocytes have been correlated with tumour risk, stage and prognosis. In patients presenting with colorectal cancer, survival was adversely affected by a lower CD4/CD8 ratio[468]. A study from Brazil showed a lower CD4/8 ratio in 15 individuals from families with a high incidence of cancer *per se* compared to a control population[486]. The same group demonstrated a lower helper/suppressor T-cell ratio in patients presenting with primary brain tumours[498]. Based on these results, GD patients with an abnormal CD4/CD8 profile may be more susceptible to malignancy.

Our GD cohort, despite being mostly treated, had a 50% reduction in the number of CD3-ve/CD56+ve NK-cells, which is in keeping with previously reported results in ERT-naïve individuals[348]. Prior publications reporting decreased NK-cell numbers in GD patients have been based on the expression of CD56 alone, which also identifies a population of large granular lymphocytes in addition to a smaller population of iNK-T cells[139;235]. CD3-ve NK-cells have a fundamental role in the lysis of cancer cells[339] and reduced numbers may predispose to a micro-environment more favourable to tumourigenesis. Convincing data supporting the benefit of NK-cells in the control of haematological cancers comes from clinical trials detailing improved survival and disease control in leukaemic patients who received an infusion of alloreactive NK-cells post bone marrow transplantation[337]. Patients with liver metastasis, secondary to gastrointestinal malignancy, have been shown to have a lower percentage of intra-hepatic CD3-ve NK cells compared to those without metastasis[500]. Infiltration of metastatic organs with NK-cells correlates with a better prognosis in bowel[501], gastric[502] and squamous cell lung cancers[503]. A reduction in peripheral blood NK-cell number or activity has been shown to correlate inversely with tumour burden in multiple myeloma and, in addition, predicts for a poorer outcome in those with DLBCL[254;504]. It has been suggested that NK-cells enhance tumour killing in DLBCL, secondary to their ability to induce death in rituximab-opsonised B-cells[504]. In a group of patient with CLL, a higher NK-cell/leukaemic monoclonal B-cell ratio was found in those with early stage disease or mutated IgH genes and predicted a longer treatment free interval[499]. Results from section 5.4.4.3 above show that patients with GD had a higher number of NK-T like cells (CD3+ve/CD56+ve) in the peripheral blood, although the absolute number of CD56 lymphoid cells was identical. Akagi *et al*. (2008) suggested that elevated blood NK-T like cells (%) conferred a worse prognosis in patients with advanced gastric cancer[505].

Several malignancies, including multiple myeloma and hepatocellular cancer, have been suggested to occur with increased frequency in patients with GD [78;130]. Prospective observational studies are required to delineate whether depleted NK numbers in GD patients are able to predict for the subsequent development of cancer or a lymphoid

clone. Due to limited patient numbers, it has not been possible to demonstrate here whether GD patients with gammopathy have a lower percentage of CD3-ve NK cells, than GD patients with normal immunoglobulins. As the GD cohort consisted almost entirely of ERT treated patients, it is unclear whether treatment-naïve individuals would share the same disturbances in immune profile. However, the number of CD56+ve lymphocytes was found to be identical in a group of patients with Fabry disease when compared to a control group, although this would have also included the detection of some large granular lymphocytes[350]. In our centre only 1 patient developed a paraprotein whilst on disease modifying therapy. Here, NK-cells in peripheral blood have been studied, but histo-pathological studies are required to demonstrate whether these findings are representative of the lymphoid infiltrate in body organs, including lymph nodes and bone marrow, of GD patients. The possibility exists that NK-cells re-distribute from the peripheral blood to body organs in GD, conferring normal immunity.

iNK-T cells, or type I NK-cells, are a unique subset which recognise glycolipid ligands presented on CD1d molecules via the T-cell receptor, which consists of a specific Vα24-Jα18 chain with preferential pairing to a Vβ11 chain[338;506]. The depletion of iNK-T cell numbers has been demonstrated in patients with cancer, including those with prostate cancer and melanoma[479;480]. Additionally, in patients with haematopoietic cancer, the percentage of Vα24+ve NK cells has been shown to be reduced in those with chronic myeloid leukaemia, lymphoma, acute myeloid leukaemia and the myelodysplastic syndrome[469]. A normal percentage of iNK-T cells was reported in one study of patients with gastrointestinal cancer [507], although the percentage reported, including in the control population, was higher than previously reported reference ranges[478;508]. The ability of expanded iNK-T populations to regress cancer has been demonstrated by several investigators in mouse models and early phase human trials, including those with advanced non-small cell lung cancer[481;509]. Both lenalidomide and thalidomide, immuno-modulatory drugs used in the treatment of multiple myeloma, have been suggested to exert their beneficial effects by increasing the number of IFN-γ secreting iNK-T cells[510]. As described above, patients with GD had an approximately 50% reduction in the mean number of peripheral iNK-T cells, based on CD3/6B11 dual

positivity. This contrasts with the findings of a Portuguese group, who reported an increase in the percentage of Vα24+ve/CD4 iNK-T cells in ERT-treated GD patients, but not in the treatment-naïve patients[4]. However, they used an antibody to Vα24, which is less specific for iNK-T cells than the 6B11 monoclonal antibody, reacting with the CDR3 epitope formed by the germ-line selection of the Vα24 and Jα18 TCRα loci. iNK-T cells only constitute 0.01-1% of all peripheral blood T-cells[478]. Therefore, these results, when compared to ours, would have been more susceptible to skewing by non-invariant NK-T Vα24+ve lymphoid populations. The identification of iNK-T cells by Vα24 positivity alone is recognised to over-estimate the population[478]. This is further highlighted by the fact that the percentage of CD4+ve type I NK-cells reported by this group (mean 0.83%) is far higher than that reported by others employing more robust flow cytometric detection protocols, including the enumeration of Vα24+ve/Vβ11+ve, 6B11+ve/Vβ11+ve or CD1d/ αGalCer binding cells[478;510;511]. The mean percentage of iNK-T cells within our control population was 0.12, which is in keeping with that reported by other investigators[508;511;512]. A reduction in IFN-γ producing iNK-T cells in individual patients with GD would compromise tumour surveillance. Again, these experiments need to be repeated in untreated individuals to ensure that these findings are not secondary to ERT. The majority of treated patients, however, had high chitotriosidase activity, skeletal disease or cytopenias, consistent with active disease.

As detailed in prior reports by Balreira *et al*. (2005), the results shown above confirm that GD monocytes have increased cell surface expression of CD1d compared to that seen in healthy controls[4;255]. Pre-treatment with the lysosomal glucocerebrosidase inhibitor, CBE, has been shown to up-regulate the cell surface expression of CD1d in healthy monocytes[4]. Balreira *et al*. (2010) reported an up-regulation of CD1d on cultured THP1 cells (monocytic leukaemia cell line) following exposure to CBE for 3 days[255]. This was statistically questionable based on low sample numbers (n=6), the inappropriate use of a parametric based t-test and the fact that in only 2/6 experiments using CBE treated THP1 cells was there evidence of increased CD1d expression. Glycolipids are known to regulate type I NK cell expansion and mouse models of several LSDs (Sandoff, Niemann Pick type I, Fabry disease, Tay-Sachs and GM1

gangliosidosis) have shown depleted iNK-T cell numbers in the liver, spleen and thymus of sacrificed animals[335]. These results are in keeping with our findings in patients with GD, despite being reported in a predominantly ERT-treated cohort. In contrast to Fabry mouse models, the number of CD4+ve and CD8+ve iNK-T cells have been found to be normal in treated and ERT-naive Fabry patients[350]. The generation of GD mouse models has been historically difficult, as enzyme deficient mice died prematurely due to epidermal water loss[513]. However, viable animal models have been established in recent years and their full immunological characterisation is awaited[514].

Lysosomal dysfunction has been hypothesised to lead to the defective expression or loading of MHC class I, MHC class II and CD1d molecules[477]. Epitope loading on MHC-I molecules was previously thought to occur only in the cytosol, although a lysosomal-dependent process has now been suggested[477]. MHC-I molecules are responsible for the presentation of tumour antigens to cytotoxic T-cells, which induce killing via the release of FasL, perforin, granzyme and nitric oxide[338]. Additionally, CD1d tetramers present endogenous glycolipid, including tumour-derived antigens, to iNK-T cells. Monocytes derived from GD patients have been shown to have elevated levels of cell surface CD1d and MHC-II, although no difference in the membrane expression of MHC-I molecules was identified[4]. ERT was suggested by the authors to decrease cell surface MHC-II levels, but to have no affect on CD1d expression. This is keeping with the results reported here where CD1d expression was significantly higher on GD monocytes.

Tumour cells can avoid immune detection by the down-regulation of MHC expression. Spanoudakis *et al*. (2009) demonstrated a reduction in both the cell surface expression and transcription of CD1d on plasma cells in individuals with relapsed myeloma when compared to those with newly diagnosed disease or MGUS[515]. The ligation of cell surface CD1d molecules has been shown to lead to the lysis of tumour cells[515]. Reduced CD1d expression is speculated to impair tumour surveillance, secondary to the inability to present antigen to IFN-γ secreting Th1 type I NK cells[336]. Gadola *et al*. (2006) demonstrated abnormal CD1d expression on the cell surface of thymocytes and

peripheral B-cells in mouse models of Niemann Pick disease, but not in the other LSD mouse models[335]. Based on RT-PCR, CD1d expression was equivalent in healthy controls (n=2), treated GD patients (n=4) and an untreated patient (n=1)[4]. However, as this analysis was performed macroscopically, based on perceived gel band intensity, and not by either optical densitometry or copy number, further studies with greater sensitivity are required to validate this result. In contrast, CD1d expression has been shown to be decreased on the cell surface of monocytes from Fabry patients, irrespective of treatment status[350]. This contrasts with the findings in the Fabry mouse model[335], but further re-enforces the differences in CD1d expression compared to that seen in GD patients. It may be that the loading of CD1d tetramers rather than the level of cell surface expression is important.

Saposins, sphingolipid activator proteins, are speculated to play an essential role in the loading of self-derived or non-self glycolipids onto CD1d molecules in the lysosomal compartment[332]. There are several potential mechanisms, hypothesised by Gadola *et al*. (2006)[335], by which the impaired loading of endogenous glycolipid onto CD1d tetramers may impair iNK-T expansion in LSDs. Firstly, endogenous lipids, such as iGB3, may be pathologically accumulated and trapped in dysfunctional lysosomes. This is in keeping with the results reported above, which demonstrated the mis-trafficking and accumulation of organelle (Golgi and endosomal) lactosylceramide to the lysosomal compartment of GD monocytes. A similar fate could be speculated to occur with glycolipid complexed CD1d molecules. Additionally, it could be hypothesised that accumulated substrate, for example glucosylceramide in GD, out-competes natural sphingolipid ligands, including iGB3, and abnormally saturates CD1d molecules in the endo-lysosomal compartment. Although these mechanisms could be postulated to lead to a decrease in type I NK cell numbers, others have argued that the chronic presentation of sphingolipid, which accumulates abnormally in GD, may lead to progressive depletion, secondary to their constant activation and subsequent exhaustion[255].

PBMCs, derived from healthy controls or GD patients, were cultured (D1-14) in the presence of IL-2 alone, IL-2 plus αGalCer or IL-2 plus iGB3. In PBMCs, CD1d was

expressed almost entirely on monocytes and was almost absent from the cell surface of lymphocytes (data not shown). Based on CD14/64 dual positivity, GD patients and healthy controls had an identical number of CD1d expressing monocytes. This is in keeping with previous reports[331]. In contrast to GD derived cultures, αGalCer led to a significant increase in the percentage and expansion of iNK-cells by D14 in experiments generated from healthy controls. In 8/11 control derived cultures, αGalCer led to a lower doubling time than any of the values recorded in the paired IL-2-only wells. Prior data have suggested that the response of individuals to expand iNK-T cells in response to αGalCer challenge is conserved over time and donors can be divided into strong or weak responders[508]. Compared to young adults (25-35yrs), healthy elderly volunteers (70-88yrs) have been demonstrated to have inferior responses in their iNK-T doubling time to αGalCer[511]. The average age of our control cohort was 37.3±7.0 years (mean±SD) compared to 39±12.4 years in the GD cohort. Compared to IL-2 wells, the addition of iGB3 did not lead to an increase in the percentage of CD3+ve/6B11+ve cells in paired cultures from control or GD patients. These findings are in keeping with those reported by Balreira *et al*. (2010) who demonstrated the proliferation of iNK-T cells in response to αGalCer but not to iGB3. In their experiments, glycolipid was presented by THP1 cells either unadulterated or matured with retinoic acid into macrophages[255]. αGalCer is a non-endogenous lipid and is known to be a more potent activator of iNK-T cells than iGB3, therefore, it is more likely to drive these cells into cell division. The injection of αGalCer into mouse models of LSDs, including Sandoff disease, Niemann Pick C and Fabry disease, has been shown to lead to impaired cytokine production (Th1 or Th2) when compared to normal mice[335]. In conclusion, GD patients have low peripheral iNK-T cell numbers, impaired responses to αGalCer stimulation and abnormal CD1d expression. Therefore it is conceivable that these abnormalities confer an elevated risk of malignancy in GD due to impaired responses to physiological sphingolipid or the inability to present tumour antigens on CD1d molecules.

PBMC experiments generated from healthy controls were supplemented with αGalCer, with or without the addition of the addition of CBE (50μM and 100μM). Treatment with CBE led to a decrease in the number of iNK-T cells on D10. CBE was not toxic to

PBMCs at any of the concentrations used (0-500μM), although selective toxicity of the iNK-T cell population could not be excluded. Further studies, using proliferation assays, such as CFSE, (carboxyfluorescein diacetate succinimidyl ester) are required to conclusively demonstrate differences in iNK-T expansion. CFSE assays have been used to confirm type I NK expansion to glycolipids in PBMC assays, in order to dismiss superior survival as a cause of increased iNK-T numbers post culture[255;508]. These results contradict the report by Ilan *et al*. (2009) who argued, at length, regarding the benefits of glucosylceramide in GD patients, and suggested an evolutionary advantage[20]. They described the beneficial effects of glucosylceramide administration in animal models of colitis, hepatitis, hepato-cellular carcinoma, melanoma and GvHD, secondary to the proliferation of iNK-T cells. Their argument assumes that glucosylceramide can be loaded physiologically onto CD1d tetramers and that iNK-T cells themselves are normal in GD. In addition, a rheostat of other sphingolipids are accumulated and depleted in GD, due to enzyme deficiency. However, the results reported above show that patients with GD have a deceased percentage of peripheral iNK-T cells that are unable to expand when challenged with αGalCer. In addition, CBE-induced enzyme inhibition blunted the expansion of these cells. This maybe due to abnormal CD1d activity[4] or intrinsic disturbances within the lymphocytes themselves, including sphingolipid accumulation. It is also currently unknown whether T-cells from patients with GD have a physiological T-cell receptor with an intact intracellular signalling cascade.

Therefore, in opposition to Ilan *et al*. (2009), it could be hypothesised that GD patients have an elevated risk of cancer secondary to numerical and functional defects in the iNK-T population. In support of this concept, patients with gastrointestinal or uterine cancer were unable to expand iNK-T cells in culture to the same magnitude as healthy controls in the presence of αGalCer[507]. This may be due to decreased iNK-T cells being a risk factor for the development of cancer or that the tumour itself suppresses this lymphocyte population. Either way, cancer surveillance is compromised. iNK-T cells have been shown to be important in enhancing tumour regression via the stimulation of NK cells[472].

NK cells were isolated from peripheral blood by immuno-magnetic separation based on CD56 positivity. Using a NK killing assay, it was demonstrated that NK-cells derived from patients with GD were not inferior in their ability to induce cell death in K562, NCI-H929 or JJN3 target cells (n=4), irrespective of augmentation with IL-2. This was despite a comparatively higher percentage of CD3-ve NK cells in healthy controls. In comparison to the CD56+ve (NK) fraction, little cytotoxicity was imparted by CD8+ve cytotoxic T-cells within the CD56-ve fraction. The killing capacity of CD56-ve cells derived from patients with GD was not inferior. The cytotoxicity of cultured PBMCs has been shown to be decreased in individuals with a family history of breast cancer[516] or cancer *per se*[517]. PBMC-induced cytotoxicity has been shown to predict for disease stage in myeloma[518] and breast cancer[343]. An 11 year follow-up study demonstrated an increased risk of malignancy in individuals whose PBMCs showed impaired cytotoxicity in killing assays[342]. GD patients were aged matched to within 10 years of healthy controls, as peripheral blood cytotoxicity has been reported to decrease with age[519]. Killing in PBMC assays is reflective of NK-cell activity and is modulated by the presence of CD56-ve lymphoid subsets and monocytes. PBMC-derived cytotoxicity assays, in addition, exploit both numerical and functional differences within individual constituents. PBMCs from GD patients were inferior in their ability to kill K562 cells at 3 different effector:target ratios. In addition, assays from GD patients displayed impaired killing of the relatively more robust NCI-H929 cell line at a ratio of 10:1. Further studies are required to explore this observation, including the possibility of an inhibitory factor being secreted by a cellular component of GD blood cells. Speculatively, individuals with an impaired PBMC cytotoxicity assay may be at an elevated risk of developing malignancy or gammopathy. Comparisons between GD patients and controls are required in order to document the number of regulatory T-cells or γδ T-cells, both being reported to have a role in tumour surveillance[336].

Although lysosomal pathology is universal amongst those with various storage disorders, GD has been the only LSD associated with an elevated risk of malignancy[78;130;132]. Many LSDs, especially those with neuronopathic involvement, lead to premature death before an age normally associated with cancer development. GD is

the most prevalent storage disorder, with large numbers of enlisted patients in data registries, supporting the capture of cancer episodes[28;130]. Prior to the introduction of ERT, males with Fabry disease typically survived to middle age. In contrast, many females with Fabry disease, due to X-linked inheritance and random lyonization, have survived for longer. Despite this, there are only isolated case reports of cancer episodes, including the development of leukaemia and bilateral renal cancer, in patients with Fabry disease[152;154].

In conclusion, GD should not only be considered a disorder of macrophages, but also of lymphocytes. GD patients, based on the data presented here, have an abnormal CD4:CD8 ratio, NK-cell subsets, peripheral blood cytotoxicity and a functionally impaired CD1d-iNK-T cell axis. Perhaps the polyclonal expansion of plasma cells in GD, secondary to chronic immune simulation, pre-disposes plasma cells to clonal expansion and these clones persist due to impaired tumour surveillance. However, it is currently unclear why this would confer a preferentially elevated risk of plasma cell neoplasms rather than non-haematological malignancies, suggesting modifying factors within the bone marrow microenvironment of GD patients. Co-culture experiments are required to assess the affect of GD lymphocytes on plasma cell expansion and other cellular components of the bone marrow, including osteoclasts. In the era of ERT, only collaborative studies can generate significant power to answer important biological questions regarding lymphoid abnormalities in untreated individuals with significant disease bulk.

6 DISCUSSION

The work presented here demonstrates novel data regarding biological disturbances in GD that may confer an increased risk of plasma cell dyscrasias including abnormalities within the monocytic and lymphoid lineages. Several clinical reports have established a high incidence of polyclonal and monoclonal gammopathy in GD, but biological models exploring these clinical observations are sparse. Abnormalities in the local microenvironment and immune profile have been described in non-GD individuals with active malignancy and in those that subsequently develop cancer. It was therefore appropriate to investigate whether similar patho-physiological disturbances occur in GD. The aim of this thesis was to explore different hypotheses and risk factors relating to the development of plasma cell dyscrasias in GD.

Firstly, in chapter 3, it was established that the RFH population of GD patients (n=9/74; 12%; mean age 59.6 years; ERT era) had a high rate of paraproteinaemia and polyclonal gammopathy. In untreated individuals macrophage-derived biomarkers, including chitotriosidase activity ($p<0.05$), S-ACE ($p<0.05$) and acid phosphatase ($p<0.05$), were significantly elevated in GD patients with gammopathy when compared with those with normal immunoglobulins. Furthermore, the Zimran severity score (ZSS) was significantly higher ($p<0.01$) in individuals with an established paraprotein than in those with polyclonal gammopathy. No difference in the ZSS was identified between those with polyclonal gammopathy and normal immunoglobulins, possibly due to limitations in the sensitivity of this scoring index. ERT led to a reduction in the absolute levels of all immunoglobulin classes (IgM [$p<0.001$], IgG [$p<0.001$] and IgA [$p<0.001$]) and stabilised the level of paraproteinaemia in all but one patient who had a paraprotein of >50g/l.

The results reported above support disease severity as being a risk factor or co-factor for gammopathy and refute the conclusions of other investigators who failed to demonstrate an association in adults[43]. These findings are in keeping with Wine *et al.* (2007) who suggested disease severity was a risk factor for polyclonal gammopathy in a paediatric population[234]. Children with early onset type I disease have been found to have high rates of polyclonal gammopathy[234], but the development of paraproteinaemia has not been reported. In the RFH cohort of GD patients, 8/9 alive

patients with a paraprotein, are currently in their 5th decade or older (4 patients 44-54 years, 4 patients 68-83 years and 1 patient aged 27 years). Unfortunately, serum electrophoresis was not routinely performed throughout their clinical care and therefore the age at which they developed a paraprotein for most is unknown. Only 1 GD patient under 30 years old currently has an M-band. GD patients may be more likely to develop clonal plasma cell disorders when exposed to disease related pathology for a long duration of time. Monoclonal gammopathy is more common in non-GD populations with advancing age[203]. Speculatively, GD related pathology, in combination with additional biological factors that are non-GD related, predisposes to early onset paraproteinaemia.

Shoenfeld *et al.* 1982 hypothesised that chronic immune stimulation in GD leads to polyclonal gammopathy and pre-disposes activated plasma cells to clonal expansion[162]. This hypothesis is supported by data presented in chapter 3, which correlates macrophage burden (biomarker data) with the presence of gammopathy. In a GD mouse model, excised lymph nodes with lymphadenitis were shown to have plasma cell infiltrates[330]. In addition, the authors reported elevated TNF-α levels, a pro-inflammatory cytokine, in the liver of sacrificed animals. In GD, it has been hypothesised that disease severity leads to enhanced antigenic stimulation, secondary to the non-physiological presentation of sphingolipids or antigen formation resulting from tissue damage. Antigen presentation has been suggested to be impaired in GD with abnormalities in CD1d molecules, MHC class II molecules and the number of dendritic cells being described[4;331].

In the cohort of patients reported here, only one individual developed a paraprotein whilst receiving ERT. In keeping with the findings of others, ERT has been suggested, to stabilise the level of paraproteinaemia but not to reduce it[43]. ERT, in some case reports, failed to prevent progression to malignant disease[81]. As individuals with GD have an increased risk of polyclonal gammopathy, MGUS and multiple myeloma, this may reflect a continuum of disease, which may be retarded by the introduction of ERT. These findings raise several issues, including financial, regarding the use of ERT in individuals with multiple myeloma or benign paraproteinaemia who do not meet traditional treatment criteria. Speculatively, polyclonal plasmacytosis may predispose to clonal expansion and may be prevented

by the early introduction of ERT. Data capture via the Gaucher registry for example is required to establish whether GD patients have a higher rate of progression from MGUS to multiple myeloma. Additionally, collaborative data is required to answer whether traditional risk factors for malignant progression, such as abnormal serum light chain analysis, the percentage of bone marrow plasma cells or the level of paraproteinaemia, hold true in GD. Whether ERT retards the rate of malignant transformation is still not known. However, some authors endorse ERT in combination with chemotherapy for the treatment of GD patients with myeloma[161], despite the lack of supporting data.

Residual glucocerebrosidase activity, genotype or prior splenectomy did not predict for the subsequent development of gammopathy. Local clinical datasets detailing spleen and liver volumes were insufficient to correlate visceral bulk with the development of immunoglobulin abnormalities. However, thrombocytopenia, a surrogate marker of sequestration, was significantly lower in untreated individuals with gammopathy ($p<0.01$). In chapter 3, we described the case of an individual who experienced a dramatic fall in the level of paraproteinaemia post-splenectomy, in keeping with a prior case report[520]. Collaborative data, including that derived from the Gaucher registry, are required to establish whether hepato-splenomegaly is a clinical risk factor for gammopathy. Glucocerebrosidase activity and genotype are considered to correlate only loosely with clinical phenotype, perhaps due to individual differences in the enzymatic activity of catabolic and anabolic sphingolipid pathways that modulate glucosylceramide load.

In vitro and *in vivo* data, in non-GD patients, have suggested that macrophages are a vital component of the myeloma bone marrow environment, support plasma cell survival and induce chemoresistance[245]. Macrophages have been shown to make up approximately 10% of cells in the trephine samples of myeloma patients[245]. Gaucher cell infiltrates of upto 50% were seen in the trephine sections of RFH patients with paraproteinaemia. Homozygous N370S individuals have been suggested to be at an increased risk of multiple myeloma compared to other genotypes and to have a high incidence of skeletal disease[52]. Non-GD individuals with haematological malignancy, including NHL, myeloma, CML, MGUS and MDS, have been reported to have elevated numbers of pseudo-Gaucher cells on bone marrow examination.

Published data has established that osteoclasts, differentiated macrophages, are supportive of plasma cell growth and survival[252;259]. These findings formed the experimental rationale for chapter 4 where biological models were used to establish whether GD-derived macrophages/osteoclasts generate a bone marrow microenvironment favourable to plasma cell expansion.

Pseudo-Gaucher cells have an identical appearance to Gaucher cells by light microscopy. Several investigators have postulated their appearance to be acquired secondary to sphingolipid accumulation from saturated glucocerebrosidase activity. The presence of these cells within the bone marrow of patients with haematological cancer leads to speculation regarding the role of pseudo-Gaucher cells and Gaucher cells in rendering the microenvironment favourable to clonal expansion. Prior data have established the infiltration and pro-cancerous role of M2 polarised macrophages in tumours. Boven *et al.* (2004) demonstrated than Gaucher cells have a phenotype of M2 macrophages[58], which are speculated to confer increased susceptibility to carcinogenesis.

Data presented in chapter 3 demonstrated normal plasma chitotriosidase activity in non-GD individuals with MGUS and multiple myeloma. Additionally, monocytic precursors from non-GD individuals with benign or malignant gammopathy were shown to have normal glucocerebrosidase activity. Therefore, non-GD individuals with paraproteinaemia fail to demonstrate the same biochemical abnormalities within the monocytic-macrophage lineage as those found in GD. Furthermore, pseudo-Gaucher cells have been shown to have a different ultrastructure and phenotype to Gaucher cells.

In chapter 4, macrophage/osteoclast cultures derived from GD patients and healthy controls were compared with respect to their ability to support plasma cell growth, survival and to induce chemo-resistance. Firstly, GD-derived peripheral monocytic cells were confirmed to have low glucocerebrosidase activity ($p<0.01$) and lysosomal dysfunction based on abnormal membrane lipid trafficking. Secondly, it was confirmed that co-culture, irrespective of monolayer origin, led to the contact-dependent proliferation and survival of NCI-H929 myeloma cells, in keeping with previous reports in non-GD patients[252]. Data presented here demonstrated that GD

monolayers did not convey either a growth or survival advantage over control monolayers to myeloma cells, based on the co-culture of NCI-H929 or U266 myeloma cell lines. These conclusions are limited by the fact that GD patients have large macrophage infiltrates *in vivo* and numerical rather than functional differences may be responsible for the elevated risk of gammopathy. This may be the key patho-physiological difference conferring plasma cell expansion in GD. Further co-culture experiments using myeloma cell lines, whose growth and survival are reliant on macrophage-derived cytokines (e.g. IL-6), are required in order to confirm the non-proliferative advantage of GD monolayers. Furthermore, co-culture experiments using primary cells, whose survival in culture is dependent on IL-6 supplementation, should be explored. Enzyme-linked immunosorbent assay (ELISA) quantification of the cytokine profile of culture supernatant might be useful in demonstrating potential differences in the levels of B-cell proliferating cytokines between GD and control-derived macrophage cultures. In addition, this hypothesis could be tested by supplementing plasma cell cultures with GD or control-derived culture supernatant. As reviewed above, published data have been contradictory, as to whether the serum levels of B-cell proliferating cytokines are elevated in GD. In addition, Gaucher cells have been shown to have weak IL-6 expression[58] but due to their accumulated bulk, a proliferative advantage on plasma cells may still be conferred *in vivo*.

Moreover, monocytes are relatively young haematopoietic cells and are unlikely to have accumulated substantial amounts of glucosylceramide during the 3 week culture period. Future experiments should be performed, using either thin layer chromatography or oil-red O staining, to clarify substrate accumulation. Speculatively, relatively young macrophages with low levels of glucosylceramide deposition may be biologically different to those with more pronounced lipid accumulation.

In chapter 4 original data are presented demonstrating that GD-derived monolayers compared to control cultures induced melphalan chemo-resistance in plasma cells lines (NCI-H929 and U226) and this was contact dependent. Melphalan exposed NCI-H929 cells derived from control monolayers exhibited higher PARP cleavage than cells harvested from GD monolayers. Furthermore, untreated NCI-H929 cells harvested from control monolayers had higher levels of the pro-apoptotic protein

Bim and higher levels of the anti-apoptotic protein Bcl-xL than that seen in cells derived from GD cultures. PUMA, another pro-apoptotic protein, was also elevated in most myeloma cell harvests derived from control experiments, compared to GD co-culture. It seems that the net disturbance in pro and anti-apoptotic proteins primes myeloma cells derived from GD culture for survival against the alkylating agent melphalan. These findings need to be confirmed in other myeloma cell lines and experiments using primary myeloma cells. Experimental limitations in this co-culture model include the use of myeloma cells with undisturbed sphingolipid metabolism. Co-culture experiments should be performed in the future with myeloma cells that have lysosomal dysfunction and substrate accumulation secondary to glucocerebrosidase deficiency. It is currently unknown whether plasma cells with dysregulated sphingolipid metabolism, perhaps due to disturbed autophagy, are more resistant to chemotherapy. Little data is available to support this hypothesis but investigators have shown that chronic lymphocytic leukaemia cells become more sensitive to chemotherapy in the presence of drugs that are known to reduce lysosomal glucosylceramide load (glucosylceramide synthase inhibitors)[457].

Speculatively, these *in vitro* data suggest that GD patients with myeloma should receive ERT in order to reduce the potentially detrimental effects of accumulated Gaucher macrophages on myeloma cell chemosensitivity. However, the addition of ERT to our co-culture system did not increase the chemosensitivity of NCI-H929 cells to melphalan in paired GD-derived experiments. This may be due to the short duration of culture experiments where the duration of exposure to ERT is insufficient to reverse lysosomal dysfunction. Further weight to this argument would be provided by the induction of chemo-resistance by GD co-culture to other chemotherapeutic agents and more novel agents, such as immunomodulatory drugs or proteasome inhibitors. Curiously, co-culture did not lead to an elevated doxorubicin IC_{50} in Gaucher-derived experiments. This may be due to differences in the mechanism of action of melphalan and doxorubicin. Due to the narrow therapeutic range of bortezomib in toxicity assays, this drug was not used in co-culture experiments. In another set of experiments, U266 cells pre-treated with doxorubicin were not rescued more effectively by GD monolayers than cultures derived from controls. Future experiments could involve the addition of chemotherapy to co-culture in order to replicate a more physiological model.

It has been previously established in co-culture experiments that plasma cells enhance osteoclastogenesis from cultured peripheral blood mononuclear cells[252]. In chapter 4 it was shown that GD macrophages, compared to control macrophages, underwent osteoclastic differentiation more readily in the presence of M-CSF and RANK-L. Results reported here demonstrate that myeloma cell line co-culture enhanced osteoclastogenesis in GD-derived cultures in a contact-independent manner, suggesting the presence of a soluble factor. In comparison, control cultures continued to generate a low number of small osteoclasts in co-culture experiments. Osteoclasts were defined as TRAP+ve cells with 3 or more nuclei. To confirm these findings, functional assays detailing bone resorption should be performed. The use of immunofluorescence demonstrating the presence of F-actin ring formation and the vitronectin receptor would also confirm osteoclastic differentiation. GD osteoclast precursors seem primed for osteoclastic differentiation and these findings should be consolidated in the future by demonstrating increased flux through intracellular pathways mediating osteoclastogenesis, including molecules upregulated by M-CSF and RANK receptor signalling by Western blotting (non co-culture and plasma cell co-culture experiments).

Future experiments should also be performed to correlate the number of osteoclasts formed *in vitro* with the induction of melphalan resistance in co-cultured myeloma cells. It is plausible that the reduction in chemosensitivity is mediated by increased osteoclast numbers rather than GD-derived macrophages. It would also be of interest to know whether *in vitro* osteoclast generation is different in GD patients with polyclonal gammopathy, MGUS or multiple myeloma. Prior data, in non-GD patients, have suggested that osteoclast numbers on bone marrow trephine examination are higher in those with myeloma than MGUS[262]. Furthermore, the same study demonstrated that patients with MGUS had more osteoclasts than healthy controls.

Recently, osteoblast-osteoclast coupling has been shown be an important modulator of plasma cell growth and survival in the bone marrow microenvironment. GD mouse models generated from the conditional deletion of *GBA1* in haematopoietic and mesenchymal tissue have recently been reported[325]. Mistry *et al.* (2010) showed

normal osteoclastic activity but disturbed osteoblastic activity based on the histo-morphometric analysis of bones in this mouse model. Early osteoblast differentiation and precursor proliferation were found to be impaired. These findings challenge the notion that osteoclastic over-activity is responsible for lytic lesions and osteoporosis in GD. The co-culture data presented in chapter 4 also conflicts with their conclusions regarding physiological osteoclastogenesis. Future co-culture experiments are required to examine the effect of GD osteoblasts on myeloma cell growth and survival.

There is already a wealth of knowledge regarding abnormalities in the lymphocyte profile, impaired tumour surveillance and cancer risk in non-GD patients. GD traditionally is classified as a disorder of macrophages with rare reports focusing on lymphocyte abnormalities. Based on the results reported in chapter 5, which includes original data, abnormalities demonstrated within the lymphoid fraction in GD patients may contribute to a microenvironment polarised towards clonal expansion.

Initially, GD lymphocytes were found to have low glucocerebrosidase activity with evidence of lysosomal dysfunction, based on the mis-trafficking of membrane lipids. Control or GD-derived lymphocytes were shown to have comparatively low enzyme activity compared to monocytes, questioning the importance of intact sphingolipid metabolism within this lineage. However, B-cells compared to T-cells from healthy controls had significantly higher glucocerebrosidase activity and therefore may be more reliant on intact enzyme activity. Whether this leads to terminally differentiated B-cells i.e. plasma cells, becoming more sensitive to proliferating stimuli in GD is speculative. Electron microscopic analysis failed to demonstrate substrate accumulation within the lymphocytes of 3 GD patients, including an untreated individual, despite the results of some authors demonstrating glucosylceramide deposition. EM analysis using immuno-conjugates to glucosylceramide or lipid analysis by thin layer chromatography of the lymphoid fraction may clarify this issue.

As reviewed in chapter 5, non-GD patients with malignancy have been shown to acquire lymphoid abnormalities in the peripheral blood or within the cancerous organ that, speculatively, contribute to a microenvironment favourable to tumour cell

survival. These include abnormalities in the absolute lymphocyte number, CD4/CD8 ratio, NK cell fraction (numerical and lytic activity) plus numbers of invariant NK-T and regulatory T-cells. The data presented here demonstrates that patients with GD have a lower percentage of CD3 positive/CD4 positive cells, a depressed CD4/CD8 ratio, a higher percentage of CD3 positive/CD8 positive cells in GD patients with gammopathy, increased NK-T like cells, a low percentage of CD3 negative NK cells and a depleted pool of invariant NK-T cells. Limitations to this analysis include the fact that comparisons were based on percentage rather than absolute lymphocyte numbers. However, GD patients had an identical absolute lymphocyte count in peripheral blood when compared to controls. Further criticisms include that the majority of patients (85%) were on ERT and the effect of disease modifying therapy on lymphocyte subset composition is currently unknown. Collaborative analysis is required of ERT naïve patients, including those presenting with severe disease, to answer this question. Paired analysis should be performed looking at the effect of ERT on T-cell subsets from individual patients over a period of time, including pre-treatment. Balreira *et al*. (2005) reported decreased CD4 positive T-cells and increased CD8 positive T-cells in the peripheral blood of patients on ERT, but not in untreated individuals compared to controls[4]. It could be hypothesised, that treated patients compared to ERT naïve individuals, due to higher peak disease burden, are more likely to develop abnormalities in their lymphoid profile. Due to the low number of untreated patients, in the analysed RFH cohort, the effect of ERT, if any, on lymphocyte subsets, could not be established.

Invariant NK-T (iNK-T) cells show remarkably plasticity and can be polarised to mediate either a Th1 or Th2 immune response. Abnormalities of iNK-T cells have been described in animal and human subjects with cancer. They normally comprise 0.01-1% of all peripheral lymphocytes and are stimulated by antigen presenting cells (APCs) harbouring glycolipid on CD1d tetramers. In the experiments described in chapter 5, GD patients had low iNK-T cell numbers that failed to expand in culture in response to αGalCer. Curiously, cultures supplemented with iGB3, an endogenous sphingolipid, failed to expand iNK-T cells derived from both controls and GD patients. This may be because αGalCer is a more potent stimulator of iNK-T cells. Preliminary experiments showed that the addition of conduritol-B-epoxide, a lysosomal inhibitor of glucocerebrosidase, blunted the expansion of control iNK-T

cells in response to αGalCer supplementation. Therefore it can be concluded that GD patients have disturbed iNK-T cell numbers and an impaired response to glycolipid stimulation that may contribute to a microenvironment conferring impaired tumour surveillance. Further experiments, using different glycolipid stimuli, are required to confirm these findings.

In addition, it was demonstrated that CD1d tetramers are expressed at a higher intensity on the cell surface of monocytic cells from patients with GD. It is unclear whether APCs from GD patients are able to recycle membrane CD1d tetramers or if they are capable of the physiological complexing of sphingolipid in the disturbed lysosomal compartment. Alternatively, naturally occurring sphingolipids may be out-competed by the preferential complexing of glucosylceramide in GD leading to a low number of iNK-T cells in vivo and an inability to load exogenous sphingolipid in culture experiments. Further work is required to explore these hypotheses. Supplementary experiments include the fluorescent labelling of CD1d molecules and time-phase studies detailing CD1d re-cycling in control and GD APCs. Additionally, although GD patients have higher expression of CD1d on the monocyte cell surface, it is unclear whether GD cells contain higher amounts of CD1d or if this represents non-physiologically re-distribution to the cell membrane. Western blotting is required to clarify the cellular content of CD1d.

NK-T cells derived from GD patients were shown to have significantly lower glucocerebrosidase activity than those derived from controls. Despite this, CD56 sorted NK-T cells from GD patients and controls demonstrated equivalence in killing assays, irrespective of pre-activation with IL-2. Furthermore the CD56-ve fraction, derived from peripheral blood of GD patients, was not inferior in the killing of immortalised cell lines. However, in paired PBMC killing assays, matched for age, GD patients compared to healthy controls demonstrated inferior killing of the K562 myeloid cell line at 3 different effector:target ratios. Criticisms of the NK cell killing assay include that the lower numbers of CD3 negative NK-cells in GD patients were not reflected in this assay but are represented in experiments utilising PBMCs. Individuals with poor prognosis breast cancer have been shown to have impaired PBMC killing activity compared to those with more favourable disease or healthy controls[343]. Therefore, it is concluded that numerical and functional abnormalities in

the peripheral blood of GD patients confer impaired tumour surveillance. Further studies are required to establish whether these findings are representative of those found in individual organ systems in GD patients, including the bone marrow.

Chapters 4 and 5 address abnormalities within the bone marrow microenvironment and lymphoid fraction respectively and provide initial insight into some of the biological mechanisms that may confer an elevated risk of myeloma in GD. *GBA1* heterozygotes have an elevated incidence of Parkinson's disease[351] but the patho-physiology behind this remains unclear. Enzyme misfolding leading to ER stress and proteasomal dysfunction has been suggested to cause cellular pathology in *GBA1* heterozygotes. However, it is unclear at present whether carrier status confers an elevated risk of paraproteinaemia, perhaps due to a similar mechanism as that present in *GBA1* heterozygotes with PD. Gaucher gene mutations are found most commonly in the Ashkenazi Jewish population (1:12-1:14 individuals) but are less common in the Sephardic Jewish population. In chapter 3, the *GBA1* analysis of 77 Jewish patients (mixed ancestry, mostly Ashkenazi [87%]) with a paraprotein, recruited from 4 different NHS trusts, was reported. In total, 8/77 (1:9.6) individuals had mutated *GBA1* alleles (Mutation [patient number]; N370s/wild type [5], 84GG/wild type [1], N370s/R496H [1] and E326K/wild type [1]). Seven patients were found to be heterozygotes with one previously unknown GD patient being identified. The data reported here does not support an increased carrier frequency of mutated *GBA1* alleles in Jewish patients with a paraprotein.

Pre-recruitment statistical monitoring calculated that 118 Jewish people with a paraprotein were required to reject the null hypothesis (power 80%, $p<0.05$, carrier frequency in Ashkenazi Jewish population 1:12). In the 4 recruiting NHS trusts we recruited 77 out of 161 (48%) individuals identified with a paraprotein. It was projected that 831 Jewish individuals greater than 50 years old, from the London boroughs served by these trusts, would harbour paraproteins. Speculatively, the majority of individuals with a paraprotein may remain clinically silent. Furthermore, *GBA1* carrier status may not confer an elevated risk of monoclonal gammopathy. Potentially, rare mutations within exons 2-4 may have been missed as sequencing was performed in exons 5-11 only. However, based on the report of Hruska *et al.* (2008), 41/261 known *GBA1* mutations are found in exon block 2-4. Allele specific

PCR and enzyme digests were used to identify the two common mutations, 84GG and IVS2+1, harboured in this region. Further criticisms of this study include that recruited individuals were of mixed Jewish descent. However, in the 55 individuals whose Jewish ancestry was known, 87% (48/55) were of Ashkenazi origin (2% unknown, 11% Sephardic Jewish). The carrier rate in Sephardic Jews of *GBA1* mutations is poorly defined but is not of the same magnitude as that found in Ashkenazi Jews. Recruitment continues in order to meet statistical targets. The acquisition of the relevant local approvals, the recruitment of patients and the time required to perform *GBA1* gene analysis is best approached in collaboration due to logistical, financial and manpower limitations. If *GBA1* carrier status leads to an elevated risk of paraproteinaemia then gene-linkage studies are required to exclude co-inheritance of a closely positioned cancer gene.

In conclusion, these studies have employed several different experimental approaches to understand why patients with GD have a higher incidence of gammopathy. Hypotheses formed were based on biological abnormalities found in non-GD patients with malignancy. This thesis reports novel data regarding clinical risk factors for the development of gammopathy in GD, the in vitro affects of GD macrophages/osteoclasts on plasma cells and lymphoid abnormalities that may confer impaired tumour surveillance (Figure 6.1). It was demonstrated that GD patients have a decreased CD4:CD8 ratio plus a low percentage of CD3-ve NK-cells and iNK-T cells. iNK-T cells derived from GD patients were unable to expand in culture to αGalCer stimulation. Furthermore PBMC killing assays derived from GD patients displayed inferior cytotoxicity to those derived from healthy controls. These abnormalities have been linked to an elevated risk of malignancy in non-GD patients. It can therefore be hypothesised that GD patients due to impaired tumour surveillance are more tolerant to clonal plasma cell expansion. Serum biomarkers of macrophage burden were predictive of gammopathy. PBMC cultures derived from GD patients were primed for osteoclastogenic differentiation when cultured alone or in co-culture with plasma cells. Osteoclasts and macrophages have been shown to support plasma cell survival in vitro. Speculatively, the GD bone marrow microenvironment, is favourable to polyclonal expansion, which favours the development of a clonal disorder on the background of chronic immune stimulation, as postulated by Shoenfeld et al. (1982). Although, GD cultures did not convey a

proliferative advantage, this was based on a numerical identical number of macrophages/osteoclasts in culture. In vivo, GD bone marrows, compared to non-GD patients, have large macrophage infiltrates. Furthermore GD cultures, compared to control cultures, protected plasma cells from melphalan induced apoptosis. As to whether *GBA1* heterozygosity confers an elevated risk of paraproteinaemia remains unanswered. Future work should build on the data and experimental models presented here.

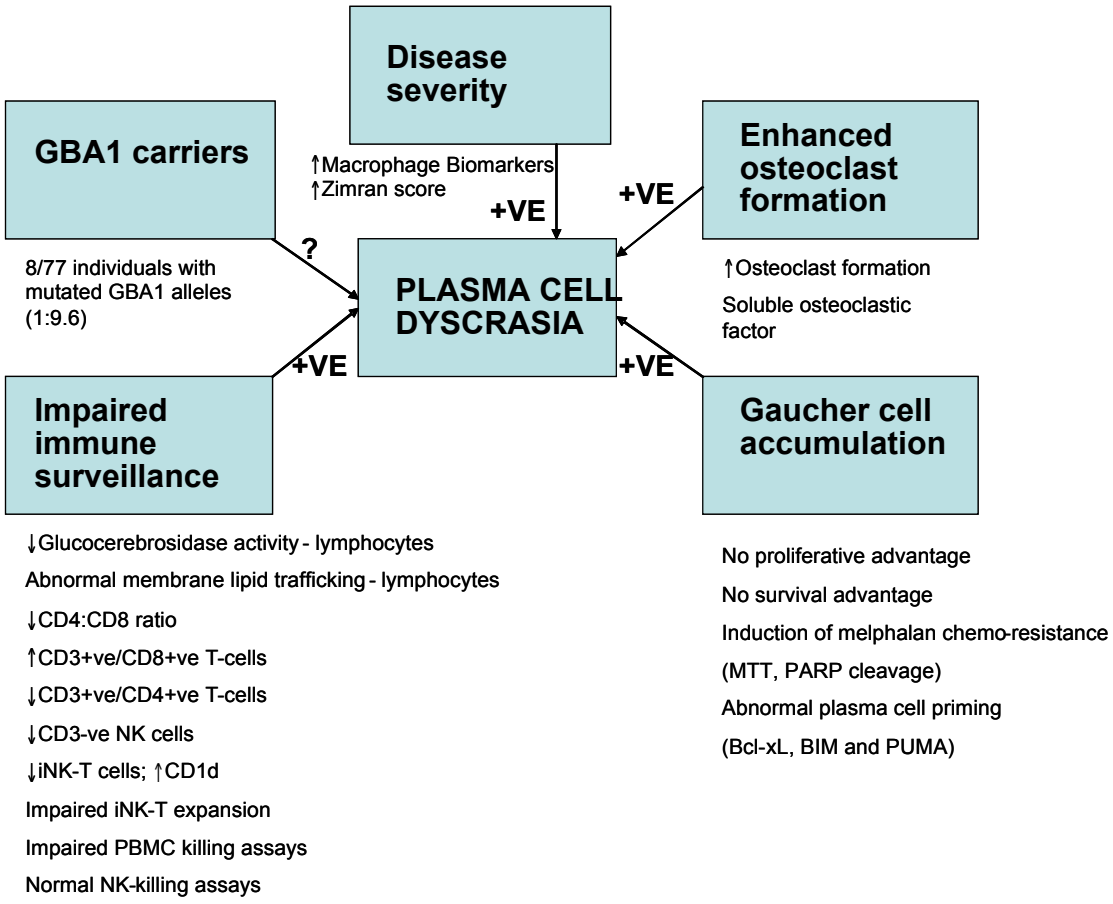


Figure 6-1. Summary of findings speculated to modulate the risk of developing plasma cell disorders in Gaucher disease.

7 APPENDIX 1

Patient Information Sheet
(Myeloma or AL-Amyloidosis Patients)

Investigation of the Linkage between Gaucher Disease and B-Cell Disorders including Multiple Myeloma

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish.

Background
Myeloma is a bone marrow cancer affecting the white blood cells of the immune system. White blood cells are responsible for attacking and destroying infection and viruses when they enter the body.

Despite 4,000 new cases every year, myeloma remains a relatively unknown cancer. The cause is unknown; exposure to certain chemicals, radiation, viruses and a weakened immune system are thought to be potential causal or trigger factors. Inexplicably, there is a higher than normal incidence in certain ethnic groups, particularly Afro-Caribbean people, suggesting that genetic factors may play a role.

AL-Amyloidosis is a condition concerning the same type of abnormal haematological cell as that seen in myeloma (plasma cell) but is its own clinical entity. Again, the cause is unknown. There are some overlapping disease similarities with those that suffer from myeloma but there are also many differences.

Gaucher disease is a rare inherited disorder in which patients lack an enzyme needed to break down certain fatty substances. This deficiency leads to the excessive storage of fatty material within the cells and tissues of the body. The most common form of Gaucher disease (type 1) affects approximately 1 in 50,000 of the general population. However, approximately 1 in 850 of the Ashkenazi Jewish population is affected and the estimated carrier status within the Ashkenazi Jewish population is 1 in 14.

For unknown reasons, patients with Gaucher disease have an increased risk of developing myeloma (estimated as 6 times more common). Approximately 1 in 10 Gaucher patients have a specific blood protein, called a paraprotein, which is found in both malignant and non-malignant conditions, including myeloma and AL-Amyloidosis.

Gaucher disease is a condition of disordered lipid/fat metabolism that leads to a particular set of observed clinical complications. Sometimes the presentation of the disorder can be extremely mild and is not picked up in extremely well patients without obvious symptoms or signs. Normal human

cells have two sets of identical chromosomes excluding sex chromosomes. Gaucher disease requires an abnormal copy of the affected gene on each chromosome. One normal copy coupled with one abnormal Gaucher gene on the other chromosome confers a 'carrier' status.

We know that patients with Gaucher disease have an increased incidence of Multiple Myeloma but we are unsure if this link is true for those who are a carrier (i.e. one abnormal gene). We are also unsure if there is an increased risk of AL-Amyloidosis in those with a Gaucher gene mutation.

As a patient with multiple myeloma or AL-Amyloidosis we would be interested in both testing your Gaucher gene status and looking at how your cells behave in comparison to normal and Gaucher patients.

What is the Purpose of the Study?
We would like to observe whether having Gaucher disease, carrier status or being mutation free affects the natural history, clinical presentation or response to treatment of multiple myeloma or AL-amyloidosis.

Why have I been Chosen?
As a myeloma or AL-Amyloidosis patient, you are suitable for inclusion in this study. Depending on your ethnic background, you may have an increased incidence of Gaucher disease.

Do I have to take part?
No. It is your decision if you want to take part or not. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You are free to withdraw your consent at any time during the study, and you do not have to give a reason for this. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will I have to do?
You will be required to fully read the information leaflet (disseminated by post or at clinical appointment) with a contemplation period of greater than 24 hours elapsing before providing signed consent. Informed consent will include demonstration of your understanding and us answering any questions that you may have in relation to the study. A consent form will then be signed in the presence of one of the clinical investigators (Dr Mehta, Dr Hughes or Dr Ayto).

We would like to collect data about your disease over a prolonged period of time (no set time length to the study). Any information required about your clinical status will be obtained from your hospital records.

Additionally we will take a few extra blood samples at either your routine clinical appointment or at an alternative time convenient to you. If you agree we could provide this either in a hospital setting or within your home. If you undergo a bone marrow examination as part of routine clinical care, we would like to take an extra bone marrow aspirate sample for testing.

If you have had a bone marrow procedure in the past, we would like your permission to perform additional tests on any stored samples in the pathology department.

If you have any questions about the questionnaire, please contact Dr Mehta, Dr Hughes or Dr Ayto (contact details listed below).

What are the possible disadvantages and risks of taking part?
We will collect research blood samples either at the time of routine blood testing or at a pre-arranged another time convenient to you. The volume of additional blood taken is relatively small and not likely to cause any side-effects. We may take the sample of blood either in a hospital setting or at your home, depending on preference.

We would like to take up-to 30mls (6 teaspoons) of blood which is a small volume that will not expose you to any additional risk. We may take a few experimental samples over time (anticipated as not more than 3 samples in 3 years) looking at different aspects of the above associations.

It is highly unlikely that you will experience any additional discomfort or inconvenience as blood will be taken by a trained professional and for existing patients mostly at the same time as your routine blood tests through the same needle.

A bone marrow research sample will only be collected if you are having this test as part of your clinical management and you have indicated this on your consent form. This specimen is small and requires no additional needle or invasive procedure. If you are undergoing a bone marrow examination then an extra 20mls (4 teaspoons) will be aspirated which again is a small volume not associated with any additional risk.

You may be diagnosed to have or to be a carrier for Gaucher disease. This is a much less serious condition than myeloma. If you are a carrier you will require no treatment but may have implications with regards to family testing. In the unlikely event you are identified as having Gaucher disease, then you will be reviewed locally at the Royal Free Hospital, one of the few national centres, in order to be counselled and to be treated. Gaucher disease is effectively treated with currently available drugs.

What are the possible benefits of taking part?
There will be no direct personal benefit and there is no financial incentive or reward. A greater understanding of the natural history of Gaucher disease and the mechanisms behind multiple myeloma or AL-Amyloid will be achieved by your participation.

What happens when the research study stops?
Our plans are to accumulate data over a long period of time and only by this can we achieve the goals of the study. Termination of the study at any point will lead to us stop collecting your data. We would like your permission to store any collected research samples in order to perform further experimental tests in the future. Consent would be re-sought if any proposed tests were not in the remit of this research study submitted and approved by our Local Ethics Committee.

Will my taking part in the study be kept confidential?
Yes. All the information about your participation in this study will be kept confidential.

Involvement of the General Practitioner/Family doctor (GP)
We will inform your GP that you are participating in this study but only if you agree and indicate this on your consent form.

Will any genetic tests be done?
Yes. Diagnosis for Gaucher disease is made by measuring enzyme activity to determine if there is a deficiency of the specific enzyme, beta-glucocerebrosidase. It is also necessary to analyse the DNA to identify the mutational changes which cause the disorders. A further genetic test we also be performed looking for common mutations in a gene that gives rise to a secretary product of Gaucher cells (pathological hallmark of Gaucher Disease) called chitotriosidase.

What will happen if I get a positive diagnosis of Gaucher Disease?
You will be referred to the National Centre at the Royal Free Hospital for assessment. The specialists there are also specialists in Myeloma and AL-Amyloidosis. If you a found to have a Gaucher gene mutation then you will receive the appropriate counselling. Gaucher disease is easily treatable with currently available drugs.

Do I have the right to object to being informed of such a diagnosis?
Yes. We consider it to be in your best interest to be informed so that you may receive the best possible treatment.

Will I be referred for re-testing outside the study?
No.

What will happen to the results of the research study?
The results of the study may be presented in scientific journals and publications and in conference presentations. They may also be shared with other participants and relevant community groups. Results of this research will be presented to the International Myeloma Foundation(UK) and the Gaucher Association.

Who is funding the research?
Funding has been given by Myeloma UK and distributed by the special trustees of the Royal Free Hospital.

Who has reviewed the study?
The study was given a favourable ethical opinion for conduct in the NHS by The Royal Free Hampstead REC.

The study was assessed independently by a Myeloma expert, and given favourable professional opinion for conduct by Dr Panos Kottaridis, Consultant Haematologist at the Royal Free Hospital.

Contact Details
If you have any questions regarding this study, please ask the doctor or nurse looking after you or contact –

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Study Number: _____

Patient Identification Number for this trial: _____

CONSENT FORM
(Myeloma Patient or AL-Amyloidosis Patient)

Title of Project: Investigation of the Linkage between Gaucher Disease and B-Cell Disorders including Multiple Myeloma

Please initial box

1. I confirm that I have read and understood the information sheet for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that relevant sections of any of my medical notes and data collected during the study may be looked at by responsible individuals from regulatory authorities or from the NHS Trust, where it is relevant to me taking part in this research. I give permission for these individuals to have access to my records. ☐

4. I agree blood samples to be taken for experimental studies including a genetic test for Gaucher disease and chitotriosidase (enzyme measure of Gaucher disease). ☐

5. I agree to an additional bone marrow aspirate sample to be taken for experimental studies if I undergo this procedure as part of routine clinical care. ☐

6. I agree to further evaluation of any prior taken bone marrow sample. ☐

7. I agree to being told my Gaucher gene mutational status and receive the appropriate counselling and follow-up. ☐

8. I agree for research specimens to be stored and understand that no additional tests will be performed outside the remit of this study unless written consent is re-sought. ☐

9. I agree to participate in this study. ☐

Please initial box

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Doctor	Date	Signature

When completed, 1 copy for patient, 1 copy for researcher site file and 1 copy (original) to be kept in medical notes

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9 PUBLICATIONS

Long-term outcomes of liver transplantation in type 1 Gaucher disease. Ayto RM, Hughes DA, Jeevaratnam P, Rolles K, Burroughs AK, Mistry PK, Mehta AB and Pastores GM. American Journal of Transplantation 10(3); 1934-1938. August 2010.

ORAL PRESENTATIONS

Ayto RM, Reed M, Baker R, Steele AJ, Wickremasinghe RG, Mehta AB and Hughes DA Title:- Gaucher derived monolayers prime myeloma cells for survival against chemo-therapeutic agents. Oral presentation. British Society of Haemaotology. April 2011, Brighton.

POSTERS

Ayto RM, Baker R North J, Reed M, Mehta AB and Hughes DA. Lymphocyte abnormalities within Gaucher disease may predispose to an elevated risk of haematological malignancy. 15th Congress of the European Haematology Association (EHA), June 2010, Barcelona

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